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A QUANTITATIVE SURVEY OF THE INVERTEBRATE BOTTOM FAUNA IN MENEMSHA BIGHT

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Extensive qualitative surveys of the marine invertebrate bottom dwelling organisms in the Woods Hole area have been carried out by Verrill (1873), Sumner, Osborn, and Cole (1911), Allee (1923), and several other investigators, but quantitative methods have not been used in the study of the fauna of this region. A study was therefore begun in order to examine in a quantitative manner the more common marine invertebrate organisms occurring within a restricted area of the bottom of the Woods Hole region.

For the purposes of this evaluation, it was decided to examine the bottom fauna of Menemsha Bight. There are two primary reasons for this choice. In the first place, Menemsha Bight is a relatively well defined body of water formed by an indentation of the western shore of Martha's Vineyard island between Gay Head and Cape Higgon, near the western end of Vineyard Sound (Chart 1). Secondly, the flounder which are captured in the Bight during the months of July, August, and September are of considerable economic importance. Therefore, it is of interest to know in what manner the bottom fauna enters into the general food cycle of the area. Either the direct or the indirect food relationships of this fish may be influenced by changes in the number and type of smaller animals living on and in the bottom. This may in turn affect the length of season when the fish are present in the region and perhaps underlie annual fluctuations which occur in their abundance.

The present survey is a preliminary attempt to investigate these problems in a quantitative manner. In addition to determining the various common species of animals present in the bottom fauna, an attempt is made to establish their numerical distribution over the area. The wet weight of each catch and dry weight of the combustible organic matter that it contained are also determined as a rough index of the possible potential food supply which the different categories of animals represent.

[1] Contribution No. 330.

[2] It is a pleasure to thank Dr. George L. Clarke for his aid in this problem. His stimulating interest was a source of much encouragement to the author.

Quantitative studies of the invertebrate marine bottom fauna in flounder fishing grounds have been made by several European investigators. The work of Petersen (1911, 1915, 1918), Jensen (1919), Blegvad (1914, 1925), and others in the Danish fishing grounds is well known. Davis (1923, 1925) has made several quantitative studies of the bottom fauna in the deep water fishing areas of the North Sea, while Idelson (1930) has carried out comparable investigations on the Spitzbergen

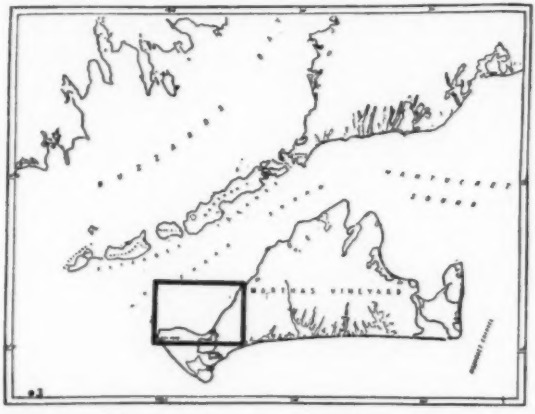


CHART 1. Map of Vineyard Sound and the adjacent islands. Menemsha Bight is the area enclosed by a heavy black line on the western end of Martha's Vineyard.

Banks. In the western hemisphere, however, the extensive quantitative studies of marine bottom fauna have been restricted chiefly to the Pacific coastal regions (Shelford and Towler, 1925; Shelford, Weese, *et al.*, 1935; Weese and MacNab, 1930) while the several investigations of bottom animals along the Atlantic coastline have been largely of a qualitative nature.

METHODS

Apparatus

Many previous investigators have found that the Petersen dredge (Petersen, 1911) was the most satisfactory bottom sampler devised to date for the purposes of their work. Preliminary trials of this instrument on sand, mud, gravel, and stony bottoms in Vineyard Sound, however, reveal that this dredge is highly unsatisfactory for quantitative sampling in this region, for it is relatively small and due to its lack of weight the jaws of the bucket frequently fail to penetrate the bottom deeply enough to obtain a sample or to capture other than organisms living upon the surface of the bottom. For these reasons, a larger and heavier dredge of the so-called "clam shell" design was purchased from the Hayward Company of New York. This instrument, made of special rust-resistant steel, weighs over 300 pounds when empty and covers a section of the bottom 101 $\times$ 56 cms. in area with its jaws open. It digs to a maximum depth of 23 cms. and holds 56 liters of material (wet sand) when level full. The single cable used in operating the bucket is tightly fastened

to and winds about a counter-balanced revolving drum in the frame of the instrument. When the cable is hauled taut, the drum rotates and closes the jaws of the bucket. The drum turns in the opposite direction and opens the jaws when strain is removed from the closing cable. Thus a releasing hook inserted into the cable at a suitable point supports the weight of the instrument, with the terminal closing cable slack and the jaws open, during the descent to the bottom.

When removing animals from samples of bottom material of the size which this dredge obtains, manual sorting of the entire contents is impractical. Therefore, the material was poured into a hopper provided with a set of three removable screens which had pore spaces of 18.0, 10.0, and 1.8 mm. respectively. The sample was washed down through the screens, each of which withheld a fraction of the catch and thus prevented the coarser material from collecting in the final screen to crush the smaller, more delicate, organisms. A comparable method of removing animals from samples of bottom material has been used with success by Petersen (1911, 1913), Blegvad (1925, 1928), and Davis (1923, 1925).

The plan of the survey

It was considered impractical, due to strong tidal currents in Menemsha Bight, to attempt a grid-work of stations over the area. Instead, the stations were made in a series of five profiles extending offshore from the 2.5 meter depth contour along a compass course, with the samples taken at definite intervals. Two additional lines of stations were made roughly parallel to the shoreline (Chart 2). The close spacing of stations along such profiles gives more accurate information on the variability of bottom types and faunal aggregations than would be obtained from a series of stations scattered over the area; and it also permits ready location and resampling at any desired station. This "contour" method has been employed by Davis (1923) who found it especially useful where the organisms existed in patches or restricted regions of the bottom.

TABLE I

Variation among 13 20-liter samples taken at 4-meter intervals in Zone 2

	Average number	Standard deviation
Total number of species	14	24%
Clymenella torquata	12	26%
Ampelisca macrocephala	19	18%

In order to establish the variations in catches which might be due to the sampling process, 13 samples of comparable size (20 liters in volume) were taken from a uniform area (Zone 2) at intervals of only 4 meters. It was found that the total number of species in each catch varied 24 per cent from the average of 14 per sample. The number of *Clymenella torquata* captured were 12 per average sample, with a standard deviation of 26 per cent; and the number of *Ampelisca macrocephala* averaged 19 per sample, with a standard deviation of 18 per cent (Table I). It is possible that these quantitative differences in the catch were due to an actual variation in the distribution of the animals over the bottom. In view of the restricted size of the region tested, however, it is assumed that the variations were caused by the sampling process itself. The average quantitative differences be-

tween the faunas of the various zones are much greater than these standard deviations of the catches in a restricted uniform area (Tables II and III). Therefore, it is believed that the quantitative data are reliable. Throughout the survey, single samples were taken at each station. Deevey (1941) found that the use of single sample stations offered satisfactory results in his studies of Connecticut lakes.

Analysis of material

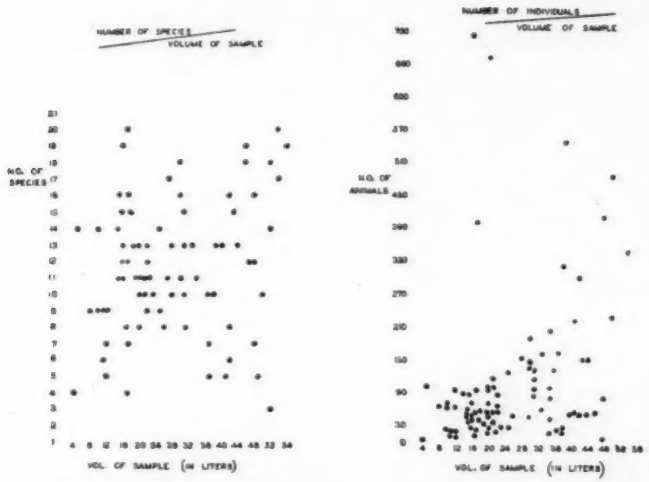
The catch from each station was brought into the laboratory where the organisms were identified and counted. The carapace of each large crustacean and the shells of all molluscs were removed and the wet weight of each catch was recorded (Davis, 1923; Berg, 1938). The estimated weight of all echinoderm tests was also subtracted from the weight of animals captured. The dry weight of the entire catch was obtained by drying the organisms to constant weight at approximately 120° C. The dried material was then ashed at approximately 650° C. By deducting the ash weight from the dry weight, an expression of the combustible organic matter present in the catch was obtained (Table III). The determination of organic content by this method is a customary procedure (Petersen, 1911; Juday, 1921; Birge and Juday, 1934).

Method of presentation of data

As in previous investigations of this nature by other investigators (Juday, 1921; Berg, 1938) it was found that the amount of bottom material obtained in the samples varied considerably, depending upon the relative hardness of the bottom and other conditions which affected the depth to which the dredge penetrated the bottom. However, there was no correlation between the size of the sample and the number of different species caught (Graph 1). In general, the larger samples of bottom material contained the greatest number of organisms (Graph 2). These facts support the assumption that the animals tend to be relatively uniformly distributed in the bottom material, at least down to the levels of the deepest grab of the dredge.

The invertebrate bottom organisms may be distributed vertically in the bottom sands in three different ways: 1. they may be restricted to a level at the surface of the bottom; 2. they may be uniformly distributed vertically throughout the bottom (down to the deepest grabs of the dredge); or 3. they may exist in greatest abundance at a level beneath the surface of the bottom material. If the first situation occurs, the quantitative data on the distribution of the organisms are best expressed on an area basis. This method has been used widely in quantitative marine studies, in spite of the variations in size of the samples obtained and although the vertical distribution of the organisms is not established. If the second situation obtains, the data should be expressed on a volumetric basis. This would eliminate variables introduced by samples of differing volume. If the third situation occurs, the choice between the area and the volumetric basis depends largely upon the numbers of animals existing between the levels of their greatest concentration and the surface of the bottom. In the present survey, the data presented in Graphs 1 and 2 support the second assumption. Therefore, the quantitative values for numbers of animals, wet weight of the catch, and the dry weight of the organic content obtained at each station have been calculated and expressed on the volu-

metric basis, using 20 liters of bottom material as the standard unit since most of the samples were of this size. The relative significance of the catch from samples of less than 10 liters or more than 40 liters in volume, when compared to that from samples more nearly approximating the standard size, is regarded as questionable, due to the large differences in volume. The data from such samples are not treated. The factor necessary to convert the data to expressions of the standard volume varied between the limits 0.5 and 2.0. Thus possible errors, should the uniform



GRAPH 1. The total number of species obtained in each sample, plotted against the volume of the sample in liters. It is apparent that there is no correlation between the size of the sample and the number of different species captured.

GRAPH 2. The total number of animals of all species in each sample, plotted against the size of the samples. In general, the larger samples contained the greater number of organisms.

vertical distribution of organisms not occur at certain stations, would be relatively small in comparison with the variations in abundance of animals from station to station and from zone to zone.

In making comparisons with other investigations, it should be remembered that this standard unit (20 liters) represents a portion of the ocean bottom 0.5 sq. meters in area, and 10.0 cms. in maximum depth. It is assumed that organisms in the bottom material below this level are of questionable importance as components of fish diet.

Inclusion of the weight of large bivalve and gastropod molluscs in the data, in view of their probable insignificance as fish food (Blegvad, 1925), would produce distortion of the results if one wishes to determine the amount of food material available to bottom-dwelling fishes. Therefore, in order to give complete values for the catches in each zone, as well as to indicate their relative significance as a source of food to fishes, the data for each zone have been expressed in two ways: 1. with all organisms included (shells and estimated weight of echinoderm tests re-

moved); and 2. with the weights of large molluscs (*Cyprina islandica, Polynices immaculata*), molluscs possessing thick shells (*Venericardia borealis, Astarte* sp.) and all echinoderms omitted. A somewhat comparable method of expressing data was used by Berg (1938) who presented his results with all organisms included, and also with molluscs omitted.

Results

The ocean floor in Menemsha Bight can be divided into five major zones arranged approximately coincident with definite depth contours. Each zone is characterized by a distinct type of bottom material, and a distinctive faunal aggregation is associated with four of these types of bottom. Table II contains a list of the

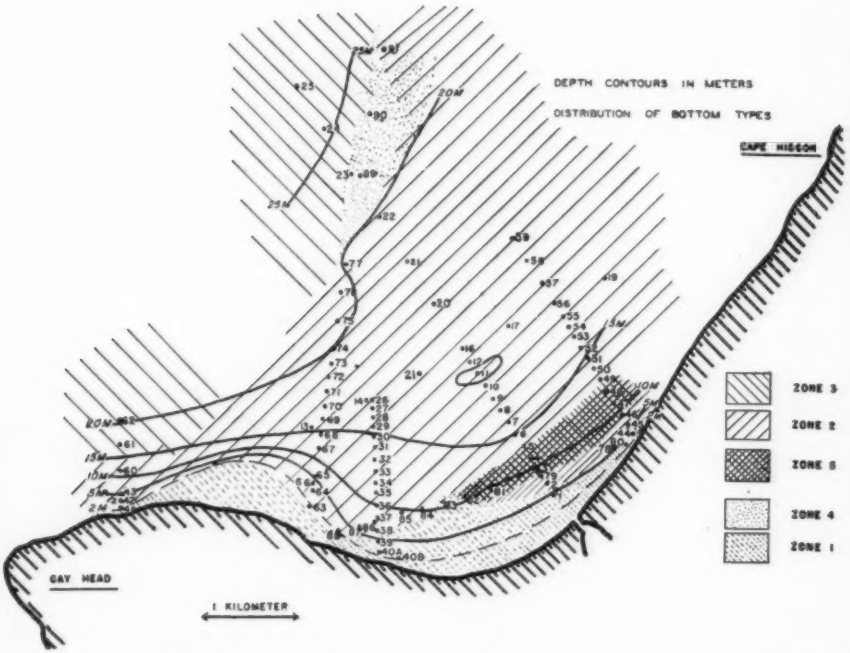


Chart 2. The number and location of each station, and the extent of the five zones in Menemsha Bight. The depth contours, in meters, are indicated by solid lines, with the exception of the 2-meter contour which is shown by a line of dashes.

catch at two stations in each zone, per standard volume of bottom material. Table III shows the predominant types of bottom (and average values for the catches) in each zone. Table IV is a list of the most common animals found in the bottom sands of Menemsha Bight and their average number in standard samples from each zone.

TABLE II

Typical catches from each zone

Zone 1.

Station 40-B

Clymenella torquata	1 specimen
Glycera dibranchiata	6 specimens
Nephthys incisa	1 specimen
Ninoe nigripes	1 specimen
Scolopolus fragilis	3 specimens
Tellina tenera	10 specimens

Station 41

Ampelisca macrocephala	1 specimen
Echinarachnius parma	1 specimen
Emerita talpoida	21 specimens
Nephthys bucera	1 specimen
Tellina tenera	19 specimens

Zone 2.

Station 26

Astarte undata	3 specimens
Clymenella torquata	27 specimens
Gammarus locusta	6 specimens
Glycera dibranchiata	6 specimens
Lumbrinereis hebes	1 specimen
Maldane sp.	3 specimens
Unciola irrorata	4 specimens
Venericardia borealis	1 specimen

Station 27

Ampelisca macrocephala	2 specimens
Clymenella torquata	9 specimens
Dolichoglossus Kowalevski	1 specimen
Gammarus locusta	3 specimens
Glycera dibranchiata	5 specimens
Lumbrinereis hebes	1 specimen
Maldane sp.	2 specimens
Nephthys incisa	1 specimen
Nephthys bucera	1 specimen
Ostrea virginica	1 specimen

Zone 3.

Station 24

Ampelisca macrocephala	31 specimens
Callocardia morrhuana	3 specimens
Cirolana concharum	2 specimens
Clymenella torquata	1 specimen
Cyprina islandica	2 specimens
Echinarachnius parma	12 specimens
Nassa trivitata	1 specimen
Polynices immaculata	1 specimen
Scolopolus fragilis	1 specimen
Unciola irrorata	1 specimen
Venericardia borealis	4 specimens

Station 25

Ampelisca macrocephala	22 specimens
Astarte quadrans	1 specimen
Cirolana concharum	1 specimen
Cyprina islandica	1 specimen
Echinarachnius parma	1 specimen
Marphysa sp.	1 specimen
Nephthys bucera	1 specimen
Polynices immaculata	1 specimen
Scalibregma sp.	1 specimen
Venericardia borealis	3 specimens

Zone 4.

The fauna of this zone was essentially similar to that of zone 3, in its quantitative and qualitative aspects.

Zone 5.

Station 48

Amphitrite ornata	1 specimen
Astarte castanea	2 specimens
Astarte undata	1 specimen
Asterias forbesi	2 specimens
Callocardia morrhuana	1 specimen
Cancer irroratus	1 specimen
Chaetopleura apiculata	2 specimens
Cirratulus grandis	1 specimen
Crepidula fornicata	692 specimens
Lepidonotus squamatus	1 specimen
Marphysa sp.	1 specimen
Pagurus longicarpus	1 specimen
Phascolosoma gouldi	2 specimens
Pinnixia cylindrica	1 specimen
Trophonia affinis	10 specimens
Venericardia borealis	19 specimens

Station 81

Arabella opalina	1 specimen
Arca transversa	2 specimens
Callocardia morrhuana	3 specimens
Cancer irroratus	1 specimen
Chaetopleura apiculata	1 specimen
Crepidula fornicata	353 specimens
Gammarus locusta	1 specimen
Lepidonotus squamatus	1 specimen
Libinia emarginata	1 specimen
Nereis virens	1 specimen
Pagurus longicarpus	7 specimens
Phascolosoma gouldi	1 specimen
Scalibregma sp.	1 specimen
Trophonia affinis	16 specimens

TABLE III

The wet weight of the catch, and the dry weight of the organic matter it contained, in the average standard-sized samples from each zone

Zone	Most numerous animals	Average wet weight of the catch in 20 liters of bottom material		Average dry weight of the organic content of the catch in 20 liters of bottom material	
		A[3]	B[4]	A[3]	B[4]
1	*Emerita Tellina Ampelisca*	5.6 gms.	1.9 gms.	0.9 gms.	0.33 gms.
2	*Clymenella Ampelisca*	27.2 gms.	10.0 gms.	2.6 gms.	1.3 gms.
3	*Ampelisca Cyprina*	164.0 gms.	9.8 gms.	29.4 gms.	1.0 gms.
4	*Ampelisca Echinarachnius*	73.0 gms.	6.3 gms.	10.7 gms.	0.5 gms.
5	*Crepidula*	364.0 gms.	360.0 gms.(?)	36.2 gms.	30.9 gms.(?)

Zone 1

Between the 2.5- and the 5-meter depth contours, the bottom is of a medium coarse yellow sand, containing a few small stones of pea size. An average standard unit of bottom material from this zone possessed five different species and a total of 32 animals of all species. *Emerita talpoida* and *Tellina tenera* were the most numerous organisms in this zone. The location of stations in this as well as in remaining zones is shown in Chart 2.

Zone 2

The coarser sands of Zone 1 graded rather abruptly (as shown by observations at stations 63–64; 38–39; 1–2; etc.) into fine mixed sands of white, yellow, and black color. This bottom material, called "Zone 2," was situated largely between the 5- and 20-meter depth contours, and extended over 80 per cent of the floor of the Bight. Annelids, small amphipods, and other organisms of known value as food for fishes were most numerous in this region, with *Ampelisca Macrocephala* and *Clymenella torquata* occurring in greatest numbers (Charts 2 and 4). The larger molluscs, however, such as *Cyprina islandica* and *Callocardia morrhuana* were more prominant by virtue of their relatively greater weight. The stations from this region which are shown in Table II illustrate the fact that the number of animals of a single common species (*Clymenella torquata*) may fluctuate 50 to 80 per cent from the average value in adjoining stations, when the number of such individuals caught is relatively low. It is seen in Table III that this zone was characterized by a heavier catch of organisms known to be of value as food for bottom dwelling fishes, than were the remaining zones.

[3] Including all organisms.

[4] With the weight of large molluscs, molluscs with thick shells, and the estimated weight of echinoderms omitted.

The number of species taken in samples from this and from the remaining zones varied from six or seven at stations 32, 33, and 34, to 19 or 20 species at stations 76, 77, 21, 70, 71, 11, 58, 59, and 52. In general, however, the regions containing the greatest number of different species were found in Zone 2. By comparing Chart 3 with Chart 4, it is evident that these regions are also generally character-

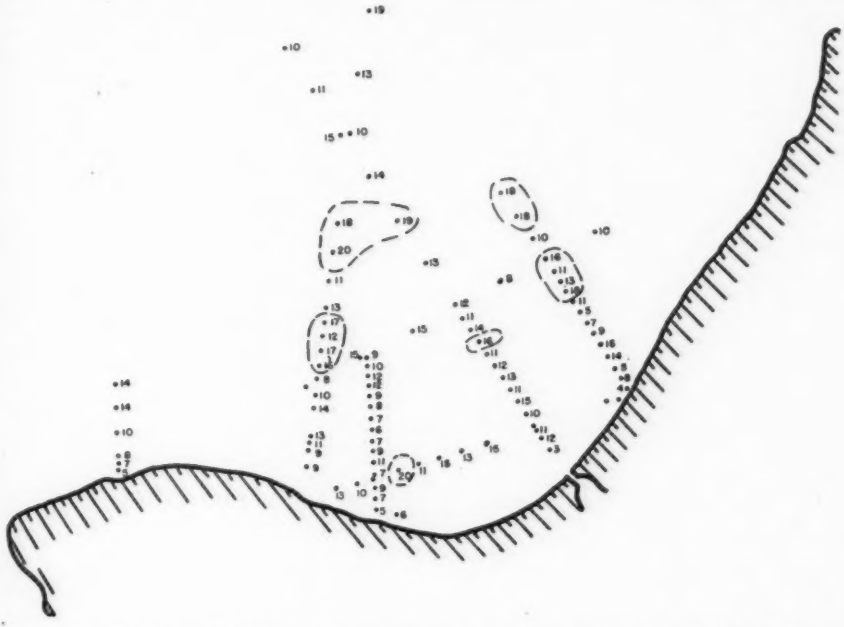


CHART 3. The number of different species captured at each station. A line of dashes encloses each region where the number of species obtained was greater than 15/sample.

ized by a great number of Ampelisca and Clymenella. In other words, in Menemsha Bight the individuals of a common species are generally more abundant in those areas where a large number of different species are found. Some of the possible factors which may determine the distribution of these organisms in Menemsha Bight will be treated in the discussion.

Zone 3

Beyond the 20-meter depth contour, the bottom was composed of loose, coarse sand, as shown by observations at stations 62, 24, and 25, which also contained shell fragments and small pebbles of various sizes. This zone contained a mixed fauna with amphipods and annelids present in each sample (Table II) but with no single species attaining numerical prominence. The large bivalve molluscs, such as Cyprina and Callocardia, composed the great bulk of each catch. It is interesting to note that *Cirolana concharum* and *Astarte quadrans* were found only at stations

in this zone; whereas several organisms (*Echinarachnicus parma, Unciola irrorata, Scolopolos fragilis*) were also found in the coarse sand bottom of Zone 1, at much shallower depths.

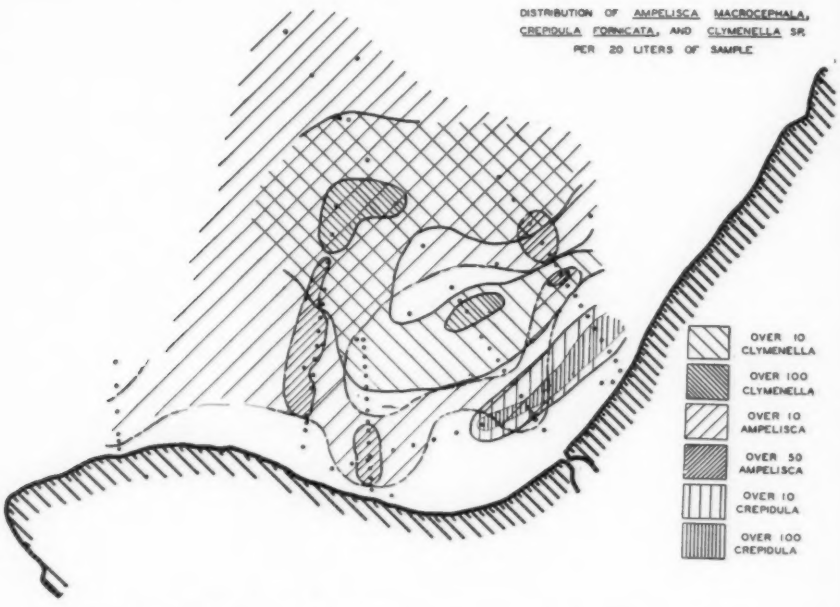

DISTRIBUTION OF AMPELISCA MACROCEPHALA, CREPIDULA FORNICATA, AND CLYMENELLA SP. PER 20 LITERS OF SAMPLE
OVER 10 CLYMENELLA
OVER 100 CLYMENELLA
OVER 10 AMPELISCA
OVER 50 AMPELISCA
OVER 10 CREPIDULA
OVER 100 CREPIDULA

CHART 4. The distribution of the three most numerous invertebrate animals in Menemsha Bight. The regions in which a common organism is most abundant are likewise characterized by a relatively greater number of different species (cf. Chart 3).

Zone 4

In the region about stations 89 and 90, a patch of medium grained white sand was discovered. There was little qualitative difference between the fauna of this zone and that found in Zone 3 (Table II). There was a slight quantitative difference, however, for the catches from Zone 3 were somewhat greater than those obtained from Zone 4 (Table III).

Zone 5

This zone was found between the 10- and the 14-meter depth contours along the eastern shoreline of the Bight (Chart 2). The bottom material of this region was of a soft fine clay, containing stones of walnut size and a considerable amount of nitrogenous matter near its surface. Mr. H. C. Stetson[5] informs me that this bottom deposit is probably of glacial origin, and contains very little marine sedi-

[5] The author is indebted to Mr. Henry Stetson, Marine Geologist at the Oceanographic Institution, for his identification of the bottom material in the major zones of Menemsha Bight.

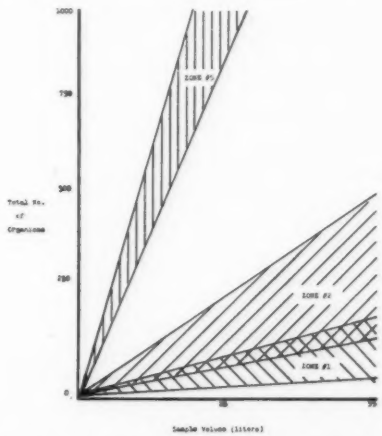
TABLE IV

The distribution of the eight most common organisms in Menemsha Bight (number of organisms/sample)

	Zone 1 15 stations	Zone 2 63 stations	Zone 3 5 stations	Zone 4 3 stations	Zone 5 5 stations
Ampelisca macrocephala	9	33	15	12	3
Clymenella torquata	0	35	1	0	0
Crepidula fornicata	0	0	0	0	423
Echinarachnius parma	3	1	4	6	0
Callocardia morruhuana	0	2	1	1	3
Cyprina islandica	0	0	4	1	0
Tellina tenera	9	0	0	0	0
Emerita talpoidia	8	0	0	0	0

ment. The bottom fauna of this region was composed of several types of crustaceans, annelids and molluscs; and the latter were predominant by number as well as by weight (Table II). *Crepidula fornicata* was exceptionally numerous and was restricted to this zone. *Trophonia affinis,* a burrowing annelid, was likewise found only in this bottom material. None of the remaining organisms was relatively abundant. The wet weight and the organic matter contained in the large numbers of Crepidula were included in the data presented in Table II although the relative importance of this organism as an article of fish diet has not yet been established. For this reason, the value for catches obtained from this zone is unusually high (Charts and Table II).

Table 4 shows the average number of the eight most common organisms obtained per 20 liters of bottom material in each zone. From these data it is possible



GRAPH 3. The total number of animals taken in each sample from the three major Zones, plotted against the volume of the sample in liters. (Fifty-five liters, the size of the largest sample, is the linear equivalent of 750 organisms.) The values for each of the Zones tend to occur within discreet regions.

to establish: 1. the numerical distribution of any one of these most abundant animals over the area; 2. the qualitative and quantitative aspects of the fauna of each zone, with respect to these organisms; and 3. the probability of capturing one or several of these organisms in a particular locality or type of bottom. The relative abundance within the zones of such organisms as Ampelisca and Clymenella and the restricted occurrence of Crepidula, Tellina and Emerita are clearly shown.

When the volume of bottom material obtained in each sample is plotted against the total number of organisms captured in that sample, it is evident that the larger samples tended to contain the greatest number of organisms (Graph 2). Graph 3 shows the relationship between the volume of the sample and the numbers of animals it contained, in the three major zones. It is interesting to note that the ratio between the size of the sample and the number of animals taken from it follows a distinctive trend in each region. The ratio ranges from 0.083 to 0.30 in Zone 1, with an average value of 0.11 (Graph 3). Similarly, the ratios for Zone 2 and 5 are 0.47 and 1.30 respectively. These figures afford a rapid means of comparing the relative abundance of organisms in each zone. In addition to their comparative value within Menemsha Bight, they offer a new method of expressing quantitative numerical relationships or comparisons between fauna of similar bottom types which may obtain in other localities. Other aspects of the bottom fauna of different regions (wet weight, content of organic matter, etc.) can likewise be compared in this fashion.

Discussion

It is apparent from a study of Charts 3 and 4 that the abundance of various species and the abundance of individuals of a single species varies considerably over the Bight. In general, the regions containing a large number of individuals of a single common species are also characterized by numerous different species. It can therefore be assumed that these regions probably offer an optimum of the conditions which determine the occurrence of these organisms. On the basis of this assumption, a closer study of these particular areas where the common animals in the Bight, such as *Crepidula fornicata* and *Clymenella torquata* are most numerous, should reveal certain of the factors which influence their abundance and distribution. Such a study is of particular importance, since many of these areas occur within that zone (2) which is by far the largest in the Bight, and which also contains the greatest numbers of animals known to be of value as food for bottom fishes.

The distribution of *Crepidula fornicata* apparently illustrates the role which the substratum may assume in determining the occurrence of an animal. This gastropod requires some relatively large hard object, such as a stone or shell, to which it fastens itself by the broad muscular foot. Such objects were found only in Zone 5, and these organisms were restricted solely to this region.

Another instance where the nature of the bottom material undoubtedly is of importance in governing the distribution of an animal, is shown by the occurrence of *Clymenella torquata* (Chart 4). This annelid is found largely in Zone 2, although a slight overlap exists to a coarser sand bottom at station 39, and to a softer bottom (which may also contain some fine sand) at stations 2, 79, 3, 4, and 5 (Charts 4 and 2). This organism constructs small tubes of the sand particles. From a comparison of the occurrence of the animal with the existence of a fine sand bottom, it

would seem that these annelids may be limited to those regions of Menemsha Bight where the bottom material contains at least a certain amount of the optimum size sand particles.

The distribution of Clymenella in the Bight also suggests that its abundance in certain regions is associated in some manner with the depth of the water. At stations 76, 77, and 21, a standard unit of bottom material contained over 100 specimens, while at station 22 they were just below this number (Chart 4). These stations border the 20-meter depth contour, which is also the outermost (deeper) boundary of the fine bottom in Zone 2. At station 11, where the depth of water increased relatively suddenly from 15 to 21 meters, the number of Clymenella is over 10 times greater than that found at adjacent stations, where the depth was 15 meters. Thus it is possible that the depth of the water, in addition to the nature of the bottom, may influence the abundance of this organism. The relative scarcity of Clymenella at stations 15, 16, and 20, however, cannot be due either to the depth of water or the nature of the bottom material. Additional factors such as prevailing tidal currents, which are at present undetermined, may also influence the occurrence of this animal.

In addition to the qualitative and quantitative variation in the fauna of different zones and of different regions of the same zone, a fluctuation was also found in the numbers and species present in the fauna of adjacent stations over a uniform bottom (Chart 3). This variation is due primarily to two causes. In the first place, many of the organisms in the Bight are widespread, but they are also sparsely distributed (Cirratulus, Cancer, Callocardia, Libinis, Polynices, Limulus, etc.). The capture of several of these animals at one location, and their absence at the next, produced a considerable fluctuation in the number of species taken in each sample. As these organisms are also relatively large, their occasional occurrence likewise produced a variation of 10 to 20 grams in the wet weight and of 1 to 2 grams in the dry weight of the organic matter contained in the catches at adjoining stations. Secondly, it has been shown that the abundance of a single common species (*Clymenella torquata*) at two adjacent stations may vary 50 to 80 per cent from an average value, when the numbers of such organisms present in the region are relatively low (Table II). This fluctuation cannot be associated with any obvious characteristics of the environment, such as the depth of the water, or the nature of the substratum. It may represent an inherent tendency of the organisms to aggregate in certain arbitrary regions of the bottom, or it may be caused by environmental factors small enough to be completely overlooked in a survey of this general nature.

The bottom fauna of Menemsha Bight was similar to that found in European flounder fishing grounds, in that molluscs, annelids, and small crustaceans were the most numerous organisms. The molluscs of the European fisheries served as the chief article of food for the bottom dwelling teleosts in the region, with the annelids and the crustaceans as the next most preferred diet (Jensen, 1919; Davis, 1923; Blegvad, 1925, 1928, 1930). However, the actual food relationships which exist between the flounder and the bottom invertebrate fauna in Menemsha Bight have not been determined at present. During two trips over the region with a commercial fishing trawler, it was found that the flounder are taken in greatest number from two widely separated belts at maximum distances of 1.0 and 3.0 kilometers from

the shore (Chart 5). It is evident that the abundance of flounder and that of Clymenella and Ampelisca (Chart 4) occur in relatively the same regions, but the actual nature of this relation remains to be established by further studies.

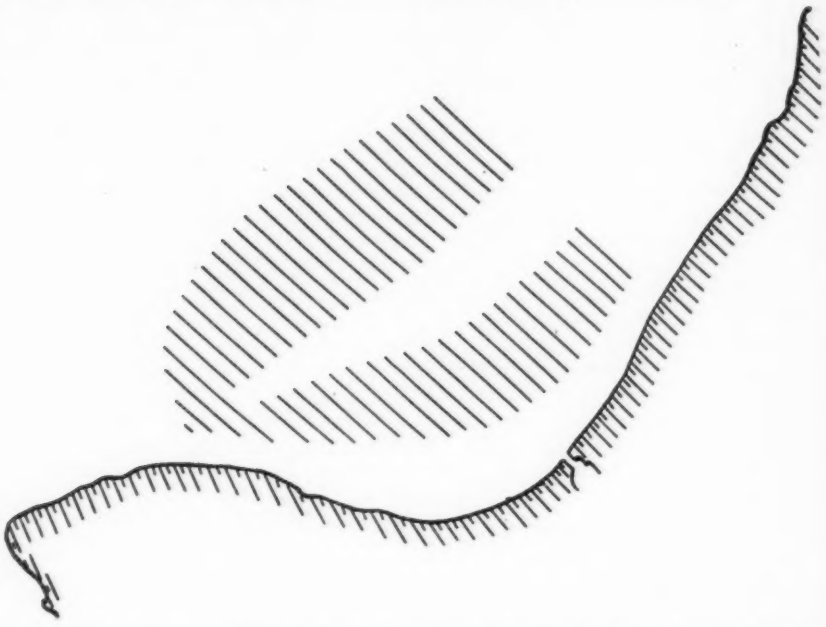


CHART 5. The diagonal lines indicate the regions of Menemsha Bight where the flounder are captured in greatest abundance. By comparing this chart with Chart 4, it is seen that these regions coincide in general with those areas where *Ampelisca macrocephala* and *Clymenella torquata* occur in large numbers.

SUMMARY

1. A dredge which is larger and heavier than those employed previously in quantitative marine bottom sampling has been used with a specially designed screening device to facilitate the removal of organisms from the relatively large samples of bottom material.

2. The bottom of Menemsha Bight, a flounder fishing grounds in Vineyard Sound, is divisable into five zones on a basis of the bottom material. Four of the zones possessed a distinct faunal aggregation.

3. The zone which extended from the 5- to the 20-meter depth contour and contained a fine sand bottom occupied 80 per cent of the area of the Bight. This zone contained the greatest quantity of the animals (per unit volume of bottom material) that are generally known to serve as food for fishes. *Clymenella torquata* and *Ampelisca macrocephala* were the most common organisms. Flounders are caught in greatest numbers in the regions where these animals are most abundant.

4. The numerical distributions of *Clymenella torquata* and *Crepidula fornicata* are correlated with distinct characteristics of the bottom.

5. In general, the regions of the Bight containing an abundance of individuals of a common species also contain a large number of different species.

6. The fauna of adjacent stations in a relatively uniform area (Zone 2) may differ considerably in both qualitative and quantitative fashion. This variation is not associated with any observed environmental differences, such as the nature of the bottom material or the depth of water. It may represent the influence of minor factors, or of mechanical aggregation.

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AN EXPERIMENTAL STUDY OF PROTOPLASMIC pH DETERMINATION.[1] 1. AMOEBAE AND ARBACIA PUNCTULATA

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Numerous attempts have been made to determine the pH of various parts of different cells. The results obtained by these investigations are not always in agreement. The literature up to 1926 is reviewed in a monograph by Paul Reiss (1926); for reference to more recent papers see Heilbrunn (1943). The picture presented in the literature, as a whole, is not a consistent one. If the question is to be settled, further work is necessary.

The purpose of this paper is to present observations on pH determinations performed by means of the injection of indicators into amoebae and the eggs of *Arbacia punctulata*. Methods are examined carefully in order to evaluate their sources of error, and the assumptions upon which their procedures are based. Some of the contradictory values in the literature are certainly due to variations in technique and the inadequacies of methods. A preliminary note has already been published, Wiercinski (1941).

This problem was suggested by Dr. L. V. Heilbrunn and I wish to express my appreciation for his advice and criticism.

Materials, Methods, and Techniques

The organisms used included *Amoeba proteus, Amoeba dubia,* and the eggs of *Arbacia punctulata. Amoeba proteus* and *Amoeba dubia* were taken from subcultures of organisms originally obtained from Dr. J. A. Dawson. These amebae were cultivated in glass finger bowls containing about 100 cc. of a modified Ringer's solution (Hopkins, 1926), to which a boiled wheat kernel and a piece of hay stem had been added. This medium was allowed to stand for a week or two until a bacterial flora developed and then the amebae were introduced. The modified Ringer's solution was made from water distilled in a metal still. Arbacia eggs were obtained as recommended by Just (1939). A sample from each batch of eggs was fertilized. If the number of eggs showed less than 98 per cent of elevated membranes the batch was discarded.

In most cases the amebae and sea urchin eggs were centrifuged before study. Centrifugation was carried out with an Emerson electric centrifuge which developed a force of about 6000 times gravity. For the Arbacia eggs, a layer of isotonic

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sucrose solution (0.73 Molar) at the bottom of the centrifuge tubes acted as a cushion. The centrifuging process was continued until the fifth layer of granules described by Harvey (1932) was plainly visible.

Clark and Lubs indicators in 0.4 per cent solution were used as noted below for injection into Arbacia eggs. Certain of the indicators were used in combination as mixed indicators. For the amebae the mixed indicators were used in a 0.2 per cent concentration. A mixed indicator has the advantage of a sharp color transformation point (pT) at a given pH (see Kolthoff and Rosenblum, 1937).

The following indicators were employed: phenol red (PR), brom thymol blue (BTB), brom cresol purple-brom thymol blue (BCP–BTB), pT at pH 6.7, brom cresol purple (BCP), brom cresol green-chlor phenol red (BCG–CPR), pT at pH 6.1, chlor phenol red (CPR), chlor phenol red-aniline blue (CPR–AB), pT at pH 5.8, 0.2 per cent methyl red (MR), brom cresol green-sodium alizarine sulfonate (BCG–SAS), pT at pH 5.6 and brom cresol green (BCG).

A comparison with microstandards was made to check the color reaction observed in the cells. The McIlvaine buffer was prepared (see Clark, 1928) and 0.25 cc. of a fresh 0.4 per cent indicator solution was added to 10 cc. of buffer. The indicator-buffer mixture was drawn into a Pyrex capillary (0.8 mm. external diameter and 0.6 mm. internal diameter), the ends sealed in a microflame and dipped in warm paraffin in order to obtain an additional seal. The microstandards were fixed on a slide about 2 mm. apart in clarite under an oblong coverslip (22 mm. × 40 mm. No. 2).

The color characteristics of the mixed indicator standards were as follows:

BCP–BTB, pT at pH 6.7. The range was taken from pH 5.7 to 7.3 at 0.2 pH intervals. At pH 7.3 the color reaction was greenish blue. The blue color diminished at pH 7.1 and 6.9. From pH 6.7 to 6.1 the color was predominantly green. At pH 5.9 the color was yellowish green and more green at pH 5.7.

BCG–CPR, pT at pH 6.1. The range was taken from pH 6.9 to 5.3 at 0.2 pH intervals. Above pH 6.1 the color increased in violet intensity. Below pH 6.1 the color appeared green and at pH 5.3 a yellowish green.

CPR–AB, pT at pH 5.8. The range was taken from pH 6.6 to 5.0 at 0.2 pH intervals. Above 5.8 the color was increasingly blue. At pH 5.8 the reaction was blue green and below pH 5.8 the color was increasingly green.

BCG–SAS, pT at pH 5.6. The range was taken from pH 6.4 to 4.8 at 0.2 pH intervals. On the alkaline side of the transformation point, this indicator exhibited a violet hue at pH 5.6 and was increasingly blue to pH 6.4. On the acid side, the color appeared to decrease in green to a yellow green at pH 4.8.

The Chambers micromanipulator was used for the microinjection of indicators into the amebae and the eggs of *Arbacia punctulata*. The material was prepared in a hanging drop over a moist chamber. Micropipettes were machine drawn from Pyrex capillary tubing (0.8 mm. external diameter) and the tip was approximately one micron in diameter when broken against the coverslip. The micropipette was washed once in the indicator solution before injections were performed. Also, by means of the Schmidtmann technique indicator particles were introduced into the amebae (see Schmidtmann, 1924; 1925).

Arbacia eggs were centrifuged in sea water and immersed in a particular medium for injection. The media used were as follows: sea water at pH 8.0 and pH 5.0;

2 parts sea water and 1 part 0.29 M. $CaCl_2$, pH at 7.6; 2 parts 0.29 M. $CaCl_2$ and 1 part sea water, pH 7.2; 70 parts sea water and 30 parts 0.53 M. NH_4Cl, pH 6.7; and 0.29 M. $CaCl_2$ at pH 6.1 and pH 7.0 (buffered with glycyl-glycine).

In most of the experiments, ten sea urchin eggs were microinjected for each medium and indicator. In all the experiments over 1000 eggs were observed. In the NH_4Cl-sea water mixtures, the eggs were allowed to remain in the solution in a shallow dish. They were then centrifuged and microinjected in the same medium.

Observations

Amoeba proteus and Amoeba dubia

The data for the uncentrifuged and centrifuged amebae are indicated in Table I.

Table I

The microinjection of pH indicators into Amoeba proteus *and* Amoeba dubia

Indicator	Number of cases	Successful injections	Color		Cytoplasmic pH	Remarks
			Indicator	in ameba		
						Uncentrifuged amebae
0.4% PR	25	15	dark red	yellow	7.0	approx. ⅕ volume or less
		0		red	7.6	approx. ½ volume
				yellow	7.0	disintegration
0.4% BCP	25	8	dark red	blue	6.6–7.0	intact amebae
				yellow	5.8	disintegration
0.2% BCG–CPR	50	4	purple	dark blue	6.6	intact amebae
				green to yellowish	6.1 5.3	disintegration
0.2% BCP–BTB	50	8	purple	blue-green	6.8–7.0	intact amebae
				yellow	5.7	disintegration
						Centrifuged amebae
0.4% PR	50	28	dark red	yellow	7.0	approx. ⅕ volume or less
		0		red	7.6	approx. ½ volume
				yellow	7.0	disintegration
0.4% BCP	25	6	dark red	blue	6.6	intact amebae
				dark blue		nucleus, pH 7.0
				yellow	5.8	disintegration

In the uncentrifuged amebae that did not disintegrate following injection, the initial reaction showed a sphering of the animal. In about 4 minutes pseudopodia were extended and the animal again appeared normal. With 0.4 per cent BCP in cases where disintegration occurred, the intact parts retained a bluish tinge of about pH 6.6. The disintegrated coagulum was a brilliant yellow color, pH < 5.8. When the indicator was released in the medium near an ameba, the movement of the pseudopodia seemed to turn the animal away from the zone of the indicator.

In the centrifuged amebae, immediately after the injection of the indicator the animal moved rapidly, redistributed the granules into the hyaline zone, and sent out many forms of pseudopodia.

Causes for failure were ascribed to injury to the ameba, overinjection of the indicator, and the toxicity of the indicator employed. In most of the cases with 0.2 per cent BCP–BTB, merely touching the ameba with the micropipette filled with indicator solution caused a violent reaction and subsequent disintegration.

Schmidtmann injection of dry indicator particles

The amebae can tolerate the injection of particles of dry indicator such as phenol red, brom cresol purple, and brom thymol blue that are not larger than one to two micra in length, width, or thickness. Comparison was made with a drop of McIlvaine buffer, pH 6.8, in which a particle of indicator was allowed to dissolve.

Forty attempts were tried and 15 of these were considered successful with phenol red. The particles colored the protoplasm quickly and were not expelled from the ameba. A churning of the interior of the animal with much pseudopodial movement was observed. However, in many cases due to injury, the ameba would disintegrate when a particle of indicator was introduced. Phenol red showed a yellow color reaction, pH < 7.0.

In two successful cases out of 20, brom cresol purple particles showed a blue color of about pH 6.6 in the protoplasm surrounding the indicator particle.

In five successful cases out of 25 brom thymol blue particles showed a green color reaction of about pH 6.6 to 6.9 in the protoplasm surrounding the indicator particle.

The microinjection of pH indicators into amebae was undertaken because of the contradictory values reported in the literature. These values are as follows: Kite (1913), pH 6.7; Needham and Needham (1925), pH 7.6; Chambers, Pollack and Hiller (1927), pH 6.8 ± 0.1; and Spek and Chambers (1934), pH 7.3. The results reported here indicate a pH of approximately 6.8.

Arbacia punctulata

Pandit and Chambers (1932) microinjected indicators into the uncentrifuged Arbacia egg in sea water. They reported a value of pH 6.8 ± 0.2 for the cytoplasm of this egg. Whenever vital dyes or indicators are used distinction must be made between the granular and hyaline portions of the cell. Pandit and Chambers did not separate the cytoplasmic components.

In the following experiments centrifuged Arbacia eggs were immersed in media of various pH and microinjected with sulfonphthalein indicator dyes. In no case was fertilization obtained after injection. The experiments were carried out at room temperature that varied from 19° C. to 24° C. In some of the injections membranes were elevated; the cortex broke down in all cases with the associated wave of reaction; the eggs appeared to swell and fifth layer granules were observed to break down with the formation of small vacuoles. In no case was a color change observed in the fat cap region. In many of the injections, the indicators seemed to be immiscible with the hyaloplasm and as a consequence membranes were formed about the droplet of indicator solution. In cases when membranes are formed the hyaloplasm may remain colorless and the color will appear as a flash. Large injections of indicator (approximately ½ volume of the egg) showed membrane formation. A smaller injection (approximately ⅕ volume of the egg or less) seemed to diffuse evenly throughout the hyaline zone.

TABLE II

Colorimetric pH determination of centrifuged Arbacia eggs in normal sea water, pH 8.0

Indicator	Number of eggs	Observed color reactions											Remarks
		Indicator		Membrane formation			Hyaline zone			SPR			
		pipette	sea water	no. of cases	color	pH	no. of cases	color	pH	no. of cases	color	pH	
PR	15	red	red	3	yellow	<7.0		yellow	<7.0				protoplasmic coagulum changed to red color
BTB	10	dark green	blue	8	yellow	6.4				1	none		
										1	green	6.8	
												6.6	
BCP–BTB, pT at pH 6.7	11	dark blue	blue	8	blue flash	6.9	2	blue	<6.7				fifth layer of granules green, pH <6.7; injected eggs turn blue
								green	6.0				
								yellow	<5.7				
BCP	10	red	blue	6	green	6.4				4	yellow	6.0	granular breakdown showed yellow color reaction
					yellow	6.0						5.8	
						5.8							
BCG–CPR, pT at pH 6.1	10	dark purple	blue	2	violet	>6.1	4	violet	>6.1	2	green	6.1	colors change to blue
							2	green	<6.1			5.7	
CPR	10	blue	dark blue	6	none		4	violet	6.6				
								green	6.0				
								yellow	5.0				
CPR–AB, pT at pH 5.8	10	purple	dark blue	6	blue flash	>5.8	4	none					color change observed following breakdown
					green	<5.8							
MR	10	yellow	yellow	4	yellow flash	>5.8	6	none					faint yellow difficult to observe
BCG–SAS, pT at pH 5.6	10	purple	dark blue	2	blue	6.2	8	blue	6.2				granular area green, pH <5.6; eggs become purple
						>5.6							
BCG	10	blue		3	blue	>5.4	2	none					eggs become purple
							5	blue	>5.4				

Surface precipitation reaction (SPR) membranes were seen to occur more often in the 0.29 M calcium chloride solutions than in the injections performed in sea water. In most cases following the breakdown of the egg, the protoplasmic coagulum changed to the pH of the surrounding medium. This was observed by the change in color of the injected indicator.

Normal sea water, pH 8.0

In this series 106 centrifuged eggs were injected. The results are summarized in Table II. The following results were obtained: PR, pH < 7.0; BTB, pH 6.8 to 6.4; BCP–BTB, pH < 6.7 to 6.0; BCP, pH 6.4 to 5.8; BCG–CPR, pH approximately 6.1; CPR, pH 6.6 to 6.0; CPR–AB, pH > 5.8; MR, pH > 5.8; BCG–SAS, pH > 5.6 to 6.2; and BCG, pH > 5.4. The probable pH as indicated in these results is in the range from pH 6.4 to 5.8.

Acid sea water at pH 5.0

This medium was adjusted with HCl and brought to equilibrium by bubbling air through the solution for 24 hours. The eggs centrifuged in normal sea water and then immersed in acid sea water often became sticky and distorted in shape. The eggs tended to clump together and often clogged the micropipette.

In this series 30 eggs were microinjected. The results are summarized in Table III. The results are as follows: PR, pH < 7.0; BCP, pH < 7.0 to 6.2; and MR, pH > 5.8.

Sea water with excess calcium

Two parts sea water and 1 part 0.29 M. $CaCl_2$, pH at 7.6

Ten eggs were microinjected with BTB. The results summarized in Table III indicate a pH of 6.0.

One part sea water and 2 parts 0.29 M. $CaCl_2$, pH at 7.2

Ten eggs were microinjected with BCG–BTB, pT at pH 6.1. The results summarized in Table III indicate a pH in the neighborhood of 6.1 to 5.9.

Calcium solutions

0.29 M. $CaCl_2$, buffered with glycyl-glycine to pH 7.0

In this series 80 eggs were injected and the results are summarized in Table IV. The results are: PR, pH < 7.0; BTB, pH 6.8 to 6.2; BCP, pH 6.4 to 6.0; BCG–CPR, pH approximately 6.1; CPR, pH 6.6 to 6.0; MR, pH > 5.8; BCG–SAS, pH > 5.6; BCG, pH > 5.4. The probable pH as indicated in these results is in the range from pH 6.4 to 6.0.

0.29 M. $CaCl_2$, pH at 6.1

Eggs immersed in this solution and returned to sea water after 35 minutes showed 100 per cent fertilization membranes when sperm suspension was added.

TABLE III

Colorimetric pH determination of centrifuged Arbacia eggs in various media

Medium	Indicator	Number of eggs	Observed color reactions											Remarks
			Indicator		Membrane formation			Hyaline zone			SPR			
			pipette	medium	no. of cases	color	pH	no. of cases	color	pH	no. of cases	color	pH	
Sea water pH 5.0	PR	10	red	red flash	1	yellow	<7.0	9	yellow	<7.0				granules showed a brilliant yellow
	BCP	10	dark red	purple flash	3	blue	<7.0	6	blue	<6.8				eggs turned yellow, pH 5.8
					1	yellow	6.2			6.2				
	MR	10	yellow	yellow	2	none	<5.8	7	yellow	>5.8	1	yellow	5.8	yellow color spread through eggs as breakdown occurred
2 parts sea water, 1 part $CaCl_2$, pH 7.6	BTB	10	dark green	dark green	5	yellow	6.0	5	yellow	6.0		yellow	6.0	
1 part sea water, 2 parts $CaCl_2$, pH 7.2	BCG–CPR, pT at pH 6.1	10	dark purple	blue	2	violet	>6.1	2	violet	>6.1	6	green	5.9	interior of SPR eggs violet, pH 6.1–6.3; all eggs turned to green in 30 to 50 seconds
						green	<6.1		green	5.9				

TABLE IV

Colorimetric pH determination of centrifuged Arbacia eggs in 0.29 M. $CaCl_2$, buffered with glycyl-glycine to pH 7.0

Indicator	Number of eggs	Observed color reactions											Remarks
		Indicator		Membrane formation			Hyaline zone			SPR			
		pipette	0.29 M. $CaCl_2$, pH 7.0	no. of cases	color	pH	no. of cases	color	pH	no. of cases	color	pH	
PR	10	dark red	orange	3	yellow	<7.0	6	yellow	<7.0	1	yellow	<7.0	
BTB	10	dark green	green	1	none		9	green	<6.8				eggs turned purple-green
								yellow	<6.2				
BCP	10	red	blue	2	green	6.4		none			yellow	5.6	
						6.0						5.4	eggs turned to yellow in 30 to 60 seconds
							2	green	6.4				
									6.0				
										4	green	6.4	
												6.0	
										2	yellow	5.6	
												5.4	
BCG–CPR, pT at pH 6.1	10	dark purple	blue	9	violet	6.1				1	violet	>6.1	in 2 minutes all eggs green
											green	<6.1	
CPR	10	dark red	red to blue	3	violet	6.6	6	violet	>6.0	1	yellow	<5.6	turned yellow, pH 5.6
						6.0							
MR	10	yellow	yellow	5	yellow	>5.8	2	yellow	>5.8	3	yellow	>5.8	eggs turn to green
BCG–SAS, pT at pH 5.6	10	dark green	green	4	blue	>5.6				6	blue	>5.6	
BCG	10	dark green	dark flash	5	blue	>5.4				5	blue	>5.4	eggs turn purple, pH 5.4

TABLE V

Colorimetric pH determination of centrifuged Arbacia eggs in 0.29 M. $CaCl_2$, pH 6.1

Indicator	Number of eggs	Observed color reactions											Remarks
		Indicator		Membrane formation			Hyaline zone			SPR			
		pipette	0.29 M. $CaCl_2$, pH 6.1	no. of cases	color	pH	no. of cases	color	pH	no. of cases	color	pH	
PR	10	red	yellow flash	2	yellow flash	<7.0	4	yellow	<7.0	3	yellow	<7.0	
				1	yellow	<7.0					none		
BTB	13	red	green	3	yellow to green	6.2 6.0	3	yellow to green	6.2 6.0				micropipette yellow from adhering coagulum and indicator
							7	yellow to green			yellow	6.0	
BCP–BTB, pT at pH 6.7	20	blue	green	12	green	<6.7 6.0	7	green	<6.7 6.0				color most pronounced in region of puncture
				1	yellow	<5.8							
BCP	10	red	green				5	green	6.2 6.0	5	green yellow	6.2 6.0 5.8	injected eggs turn to yellow, pH 5.8
BCG–CPR, pT at pH 6.1	10	dark purple	blue				6 4	violet violet	>6.1 <6.1		green	5.8	granules were yellow, pH 5.4
CPR	10	red	violet	3	violet	6.0				6	yellow	<5.6	appeared dark blue, pH >7.0
				1	blue to violet	6.2		violet	6.0				
CPR–AB, pT at pH 5.8	20	blue	blue	19	blue	>5.8				1	blue green	>5.8 <5.8	
MR	10	yellow	yellow flash	6	yellow	>5.8				4	yellow	>5.8	
BCG–SAS, pT at pH 5.6	10	dark blue	blue				10	blue	6.2 6.0				eggs absorbed indicator, blue, pH 6.0–6.2
BCG	10	dark green	blue				6 4	blue blue	5.6 5.6		green	5.6	change to yellow, pH 4.0

TABLE VI

Colorimetric pH determination of centrifuged Arbacia eggs in 70 parts sea water, 30 parts 0.53 M. NH_4Cl, pH 6.6

Indicator	Number of eggs	Observed color reactions											Remarks
		Indicator		Membrane formation			Hyaline zone			SPR			
		pipette	sea water, NH_4Cl, pH 6.6	no. of cases	color	pH	no. of cases	color	pH	no. of cases	color	pH	
PR	10	red	yellow flash	2	none		6	yellow	<7.0	2	yellow	<7.0	yellow ring of color circumscribed egg
BCP	10	red	blue	2	yellow	5.8	4	green	6.0				granular area purple
				3	green	6.4	1	blue	6.8				
					yellow	5.8		green	6.4				
								yellow	5.8				
BTB	10	red	green				9	green	6.8	1	yellow	<6.4	
									6.4				
BCP–BTB, pT at pH 6.7	10	dark blue	green ?	2	green	<6.7	5	green	<6.7				
							3	green	<6.7				
								yellow	<5.8				
CPR	10	dark red	dark blue ?	5	blue	6.6	5	blue	6.6				
						6.0			6.0				
BCG–CPR, pT at pH 6.1	10	purple	dark	3	blue	>6.1							
			blue	7	blue flash	6.1							
CPR–AB, pT at pH 5.8	12	purple	dark blue	5	blue	>5.8	7	blue	5.8				
MR	10	yellow	yellow flash	2	yellow	6.0	8	yellow	6.0				
						5.8			5.8				
BCG–SAS, pT at pH 5.6	10	green	light green				8	blue	6.0	2	blue	6.0	some eggs absorbed indicator, turned green, pH <5.6
BCG	10	dark purple	blue	4	dark blue	>5.4	6	dark blue	>5.4				

The eggs seemed to withstand much handling and manipulation. However, some batches of eggs showed a tendency to stick together when immersed in pure calcium solution.

In this series 133 eggs were microinjected and the results are summarized in Table V. The results are: PR, pH < 7.0; BTB, pH 6.2 to 6.0; BCP–BTB, pH < 6.7 to 6.0; BCP, pH 6.2 to 6.0; BCG–CPR, pH approximately 6.1; CPR, pH > 5.6 to 6.2; CPR–AB, pH > 5.8; MR, pH > 5.8; BCG–SAS, pH about 6.0 to 6.2; and BCG, pH > 5.6.

Ammonium chloride-sea water

Seventy parts sea water, 30 parts 0.53 M. NH_4Cl, pH at 6.6

Different batches of eggs were allowed to remain in this medium for about 6 hours. An increase in the size of the fat cap occurred, and close to 98 per cent fertilization was obtained. According to Jacobs (1922) ammonium chloride is believed to cause an alkalinization of the protoplasm.

In this series 112 eggs were microinjected and the results are summarized in Table VI. No color change was observed in the fat cap region. The results are as follows: PR, pH < 7.0; BCP, pH 6.8 to 5.8; BTB, pH 6.8 to 6.4; BCP–BTB, pH < 6.7; CPR, pH 6.6 to 6.0; BCG–CPR, pH > 6.1; CPR–AB, pH > 5.8; MR, pH 5.8 to 6.0; BCG–SAS, pH 6.0; and BCG, pH > 5.4. The probable pH as indicated in these results is in the range from pH 6.6 to 6.0.

Protein-lipid error of indicators

The presence of proteins and lipids is a well recognized source of error in the colorimetric determination of pH. It was of interest to test the interference of protoplasmic and lipid substances of Arbacia eggs upon pH indicators. This was done in the following manner:

The hyaline and granular halves of Arbacia eggs were obtained by means of high speed centrifugation. The respective halves were removed from the centrifuge tube with a pipette and put into separate test tubes. The portions were washed by shaking in 2 cc. of 0.35 M. sodium citrate-citric acid buffer at the desired pH. This mixture was centrifuged at low speed and the supernatant fluid was decanted. Two cc. of fresh buffer was added. The proportion was approximately $\frac{1}{16}$ cc. of egg substance (hyaline or granular halves) to 2 cc. of buffer. The hyaline or granular buffer mixture was transferred to a small mortar and ground with a pestle. The ground mixture was tested electrometrically with a Beckman pH meter and colorimetrically with indicators. The colorimetric determination of the ground hyaline-buffer mixture was made on a white spot plate. The proportion was $\frac{1}{16}$ cc. of 0.02 per cent indicator to 1 cc. of mixture. The granular halves were too dark with pigment to determine a colorimetric error.

The results are shown in Table VII. Phenol red showed a colorimetric error of pH -0.1 to $+0.02$, brom cresol purple ranged from pH -0.17 to $+0.22$, and brom thymol blue pH $+0.28$ to $+0.92$.

Colorimetric pH error was also determined with dried Arbacia substance. This was added to a 0.1 N HCl solution diluted with distilled water to various pH intervals. The proportion was 5 cc. of acid and 0.05 g. of Arbacia substance.

TABLE VII

Protein-lipid error of pH indicators determined with hyaline Arbacia substance (Na Citrate-Citric Acid Buffer)

Indicator	Electrometric pH	Colorimetric pH	Electro-Color pH
PR	7.02	7.0	+0.02
PR	7.19	7.2	−0.01
PR	7.30	7.3	0.00
BTB	6.48	6.2	+0.28
BTB	6.67	6.1	+0.57
BTB	6.83	6.1	+0.73
BTB	7.02	6.1	+0.92
BTB	7.19	6.5	+0.69
BTB	7.39	6.7	+0.69
BCP	5.64	5.5	+0.14
BCP	5.82	5.6	+0.22
BCP	6.04	6.0	+0.04
BCP	6.18	6.1	+0.08
BCP	6.48	6.1	+0.08
BCP	6.67	6.5	+0.17
BCP	6.83	7.0	−0.17

The results are shown in Table VIII. Phenol red showed a colorimetric error of approximately pH + 0.3, brom thymol blue approximately pH + 0.1 to + 0.4, and chlor phenol red approximately pH + 0.2 to + 0.4.

TABLE VIII

Protein-lipid error of pH indicators determined with dried Arbacia substance (0.1 N HCl Solution)

Indicator	I Electrometric pH	II Colorimetric pH	I–II Electro-Color pH
PR	7.30	7.0	+0.3
BTB	6.43	6.3	+0.13
BTB	6.80	6.4	+0.4
CPR	6.12	5.7	+0.42
CPR	5.78	5.5	+0.28
CPR	5.72	5.5	+0.22

DISCUSSION

Chambers (1929) and his coworkers have injected pH indicators into many types of cells. They arrive at a value of pH 6.8 ± 0.2 for the cytoplasm of all of them. If this is correct, it is a most interesting generalization.

In this country and in England, the data presented by Chambers have been universally accepted. However, in the European literature other pH values are reported as follows: Reiss (1928) cytoplasm of the *Paracentrotus lividus* egg, pH 5.6, Vles and Vellinger (1928) cytoplasm near the pigment granules in Arbacia, pH 5.5 ± 0.3, Spek and Chambers (1934) hyaloplasm of *Amoeba dubia,* pH 7.3,

Reiss and Gersch (1936) macromeres and micromeres of *Aplysia limacina* somewhere between pH 6.0 and 7.0, and Raven (1937) cytoplasmic inclusions of Chaetopterus, Nereis, and Aplysia eggs are acid and basic, and the hyaline zone is not colored by indicators.

Work with amebae indicated that these organisms were able to tolerate the microinjection of minute quantities of sulfonphthalein indicator dyes (see Reznikoff and Pollack, 1928). Phenol red was the least toxic of all the indicators used. In *Arbacia punctulata* eggs, the microinjection of sulfonphthalein indicator dyes was followed by a wave of reaction in which a breakdown of cortical and fifth layer granules occurred. In most of the cases the indicator was found to be immiscible with the hyaloplasm as seen in the numerous instances of membrane formation about the zone of injection.

The results show that the protoplasm of different types of cells does not have the same pH but that, for the cells examined, the pH lies within a range from pH 6.0 to 7.0. Ameba hyaloplasm appeared to be more alkaline than the hyaloplasm of sea urchin eggs.

In all the media, the observed colorimetric pH values for the Arbacia hyaloplasm were in the range from pH 5.8 to 6.8. In agreement with Chambers (1929) for other eggs, the nucleus was found to have a pH above 7.0. After breakdown of the fifth layer of granules, the pH of the granular material was found to be 5.4. Upon complete cytolysis, the cytoplasmic pH becomes more acid. After the external medium invades the cytolyzed egg, the internal pH is similar to the external pH. Injections made in the heavy granule area gave inconsistent and uncertain results.

The pH of the surrounding medium does not appear to have much effect on the hyaloplasm pH (also see Chambers, 1928). The pH value for individual measurements showed some variation but this was not related to the pH of the surrounding medium. Thus, when Arbacia eggs were immersed in sea water at pH 8.0 as compared with sea water at pH 5.0, no marked differences were observable in the initial pH reading. Moreover, when eggs were immersed in sea water containing NH_4Cl under conditions in which alkalinization of the protoplasm is believed to occur, no such effect could be observed in the hyaloplasm. In the original observations of Jacobs (1922) of alkalinization in such a solution, the observations were made with uncentrifuged eggs in neutral red solution which stains the granules. Chambers (1928) discusses the granular pH in this regard. Presumably, the granular pH, that is to say the pH within the vacuoles of the granules, is affected by the ammonium ion. See Harris (1939) for a description of granular vacuoles in Arbacia eggs.

Errors of visual color determination in the microscopic field are due to the "personal equation" of the observer, rapidity of the color change, and light conditions. Colors can be altered by the reflected colors of their surroundings, by the character of light in which they are observed, the condition of rest or fatigue of the eye, and the presence of after image on the retina (see Maerz and Paul, 1930).

Inasmuch as cells contain salt and protein plus lipid, there is a possibility of salt and protein error. However, observations indicate that these apparently are not great except in the case of protein-error of Arbacia substance for brom thymol blue as indicated in the results.

In this work, distinction between granules and hyaloplasm has been attempted by centrifuging. This has not been done before in the colorimetric study of protoplasmic pH determination. The factor of indicator volume to cell volume in the quantitative study of pH with the colorimetric method is important. A method has not yet been worked out where it is possible to inject the same amount of indicator into several successive cells. This procedure is necessary to obtain more than approximate values.

The toxicity of indicators and the chemical make-up of standard indicator solutions must be considered. A 0.1 N NaOH solution is the basic solution in which most sulfonphthalein indicators are dissolved. Seemingly, the introduction of an alkaline solution into the cell would give more alkaline pH values, especially when the indicator solution is approximately ⅕ to ½ of the cell volume.

Conclusions

Within the limitations of the methods used the following values are believed to represent the approximate protoplasmic pH for the various cells studied:

Amoeba proteus pH 6.8 ± 0.2
Amoeba dubia pH 6.8 ± 0.2
Arbacia punctulata eggs pH 6.2 ± 0.2

Protein-lipid errors of indicators determined with Arbacia substance vary from pH -0.17 to approximately $+0.9$ for various indicators.

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THE INDUCTION OF LARVAL MOLTS IN DROSOPHILA

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It has recently been shown (Bodenstein, 1943) that a hormone produced by the ring gland controls the growth of the imaginal organ anlagen in Drosophila during the larval period, as well as the growth of purely larval organs. Since molting is mainly a process by means of which the larval insect grows, one might expect it to be governed by the same principle, yet previous experiments were unable to shed any light on this point. It is the purpose of this investigation to provide convincing evidence that the molting of Drosophila larvae is under the control of the larval ring gland.

Material and Methods

The larvae of Drosophila molt twice before they pupate. At each molt they shed their mouth parts, together with their cuticula. The mandibular hooks of the mouth armature, characteristic in size and shape for each larval instar, were used as a criterion for molting. For the experiments the larval head segments, including the complete mouth armature, were cut off from larvae shortly after their emergence from the egg and from 2nd instar larvae. Special care was taken not to include the brain and attached ring gland in the isolated head segments. The brainless head pieces were then transplanted into the body cavity of adult flies or into mature larvae. The transplants, either alone or together, were put with several ring glands into the body cavity of the adult hosts. The hosts were dissected at different times after the operation; the transplants removed *in toto,* stained with orcein, cleared and mounted in diaphane. The number of mandibular hooks present in the transplant could easily be observed in these preparations. In many cases one could also use the spiracles as an indicator for molting, because these structures are shed at each molt and are characteristic for each instar. The majority of experiments was performed on *Drosophila virilis* (wild stock). The experimental animals were kept at a constant temperature of 25° ± 0.5° C.

I am greatly indebted to Dr. L. C. Dunn and Dr. T. Dobzhansky for many stimulating discussions and for their continued interest in the work.

Experiments

The transplantation of larval head segments into adult male hosts

In a series of experiments, larval head segments without brain and ring gland, but containing the complete mouth apparatus, were transplanted into the abdomen of adult male flies and dissected at different times after the operation. These experiments comprise three different experimental groups. In one group the head

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segments of 1st instar larvae not older than 4 hours were used. In the second group the head segments of the larvae were somewhat older but their age never exceeded 10 hours. In the third group head segments of 2nd instar larvae were transplanted. The experiments are summarized in Table I. They show that none of the transplanted head parts has molted, since only one pair of mandibular hooks is present. Usually there are some brownish spots in the grafts, while the rest of

TABLE I

Transplantation of larval head segments into the abdomen of adult virilis male flies

Age of transplanted head segment	Number of cases	Days transplant remains in host	Number of cases where transplant has only one pair of mandibles, i.e. has not molted
1st instar (4–6 hrs. old)	1	4	1
	4	7	4
	3	8	3
	4	11	4
	10	14	10
	1	15	1
	13	16	13
	3	17	3
	7	18	7
	3	19	3
1st instar (ca. 10 hrs. old)	4	16	4
2nd instar	12	8	12
Total number of cases	65		65

the tissue maintains its larval color characteristics. Grafts which remained very long in the host frequently show some brownish cuticle coloration, which resembles the color of young pupae. Whether this color is an indication of pupation is, however, questionable. Strong muscle contractions of the grafts at dissection demonstrate very well their living condition.

The transplants undergo certain other changes while in their adult hosts. It is often noticed that the cuticle between two adjacent segments becomes somewhat darkened in transplants left for 2 or 3 days in their hosts (Fig. 1). If left longer in the host the larval head pieces exhibit a typical behavior. A fine epithelial membrane begins to appear on the proximal end of the transplant where the body wall of the larvae was cut. This epithelium grows gradually, creeping around the outside of the larval cuticle of the head part until finally the whole transplant is enveloped by a fine transparent tissue sac. Figures 2, 3, 5 and 6 show this condition for some selected cases. It should be explained however that the completeness of this overgrowth does not entirely depend upon the time the implant remains in the host, for in some cases the overgrowth might have been enveloped only one-half, in others the whole of the transplant, although all remained for the

same length of time in the host. The tissue envelope apparently represents the regenerating wound epithelium of the larval epidermis. Its cells are very large and extremely flat, with large nuclei containing polytene chromosomes. In life the space between the epithelium mantle and the larval cuticula appears transparent and empty. In sectioned material, however, it seems to be filled with a laminated deposition (Fig. 6). Whether this deposit is chitin has not been determined.

Piepho (1938) in his studies on Galleria (wax moth) has observed the same type of behavior. He transplanted small pieces of caterpillar skin into the abdomens of other caterpillar hosts, and found that an epidermal cover (*Umwachsungshypodermis*), originating from the epidermis of the transplant, gradually enveloped the implant. In a careful histological study, Piepho found, moreover, that the differentiation achievements of this enveloping tissue sheath may vary according to the hormonal situation of the host.

The transplantation of larval head segments together with ring glands into the abdomen of adult flies

Head segments of 1st and 2nd instar larvae without brain and ring gland were transplanted into the abdomen of adult flies. Unlike the former series, however,

TABLE II

Transplantation of head segments together with ring glands into the abdomen of adult flies

Age of transplanted head segment	Host and transplanted ring gland	Number of cases	Days transplant remains in host	Number of cases where transplant has formed				
				1 pair of mandibles	2 pairs of mandibles	3 pairs of mandibles	spiracle of 2nd inst.	spiracle of 3rd inst.
1st instar (4 hrs. old)	v. 2RG	4	8	3	1			
1st instar (4 hrs. old)	v. 2RG	2	15	1	1			
1st instar (4 hrs. old)	v. 2RG	6	16	4	2?			
1st instar (4 hrs. old)	v. 2RG	1	5	1				
1st instar (4 hrs. old)	v. 2RG	2	7	2				
1st instar (6 hrs. old)	m. 2RG	3	7		2	(1?)		1
1st instar (10 hrs. old)	m. 2RG	9	15	2(4?)	2	(1?)	3	(1?)
2nd instar	v. 3RG	6	8	4	2			3(1?)
2nd instar	m. 3RG	3	6	2	1			
2nd instar	m. 3RG	3	8	2	1			3
Total number of cases		39			14 positive			

v. = virilis; m. = melanogaster.
RG = Ring gland.
Number in parentheses indicates number of cases where the mandible or spiracle number formed is not clear.

two or three ring glands from mature larvae were transplanted simultaneously with the larval heads into the same hosts. Not only adult males but also adult virilis females were used as hosts. In many cases adult melanogaster females were employed as hosts, for it had been found previously (Bodenstein, 1943) that the ring gland effect in melanogaster females was much stronger than in virilis females. The results of these experiments are summarized in Table II. They show that in the presence of ring glands the larval head segments can be induced to molt, as indicated by the presence of two pairs of mandible hooks in one transplant. Figures 7 and 10 show a 1st instar transplant and Figure 8 shows one 2nd instar transplant after molting has been induced by the grafted ring gland. In only one case (one other questionable), molting had occurred twice. Although only two pairs of mandibles were found in this case, one pair belonging to the 1st and the other to the 2nd instar, a spiracle typical for the 3rd instar was present, proving that the second molt had taken place (Fig. 11). As far as the molting competence of mandibles and spiracles is concerned, it seems that the spiracles react somewhat more readily to the molting hormone of the ring gland than the mandibles. This is indicated by the fact that of six 2nd instar transplants only three possessed two pairs of mandibles, while all six heads had formed 3rd instar spiracles.

Apart from their molting, the developmental behavior of these transplants is very similar to that discussed in the foregoing section. They apparently form the same type of regenerated wound epidermis cover which envelops either part or the whole of the head segments. The cuticular color of the graft under the epidermal

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PLATE I

FIGURE 1. Head segments of 2nd instar larva transplanted into an adult virilis male host 8 days after the operation. Note darkening of segment borders.

FIGURE 2. Head segments of 1st instar larva transplanted together with two larval ring glands into an adult virilis male host. Transplant remained 15 days in host but has not molted. Note that an epidermal sheath has enveloped about one-half of the transplant.

FIGURE 3. Head segments of 1st instar larva transplanted into an adult virilis male host 14 days after the operation. Note that an epidermis sheath has completely surrounded the transplant.

FIGURE 4. Head segments of 1st instar larva transplanted into an adult virilis male host 14 days after the operation. An epidermis sheath has almost completely overgrown the transplant, leaving only the tip of the head free. At the proximal end of the transplant note the two quite extensively developed eye discs.

FIGURE 5. Section through head segments of a 1st instar larva, transplanted into an adult virilis male host 17 days after the operation. The epidermis has surrounded only one-half of the transplant. Note the large nuclei(n) in the epidermis cover.

FIGURE 6. Section through head segments of a 1st instar larva transplanted into an adult virilis male host 18 days after the operation. The epidermal envelope covers the whole transplant. Note the large nuclei(n) of the epidermal envelope and also the striated material between the transplant and the epidermal sheath.

FIGURE 7. Head segments of 1st instar larva transplanted into mature larvae 5 days after the operation. Three pairs of mandibular hooks, indicating that the transplant has molted twice.

FIGURE 8. Head segments of 2nd instar larva transplanted into a mature larva 8 days after the operation. The transplant has molted but once, as indicated by the presence of only two pairs of mandibular hooks.

FIGURE 9. Head segments of 1st instar virilis larva transplanted into a mature pseudoobscura larva 7 days after the operation. Note three pairs of mandibular hooks, the third and largest pair somewhat proximal to the others. The transplant has thus molted twice.

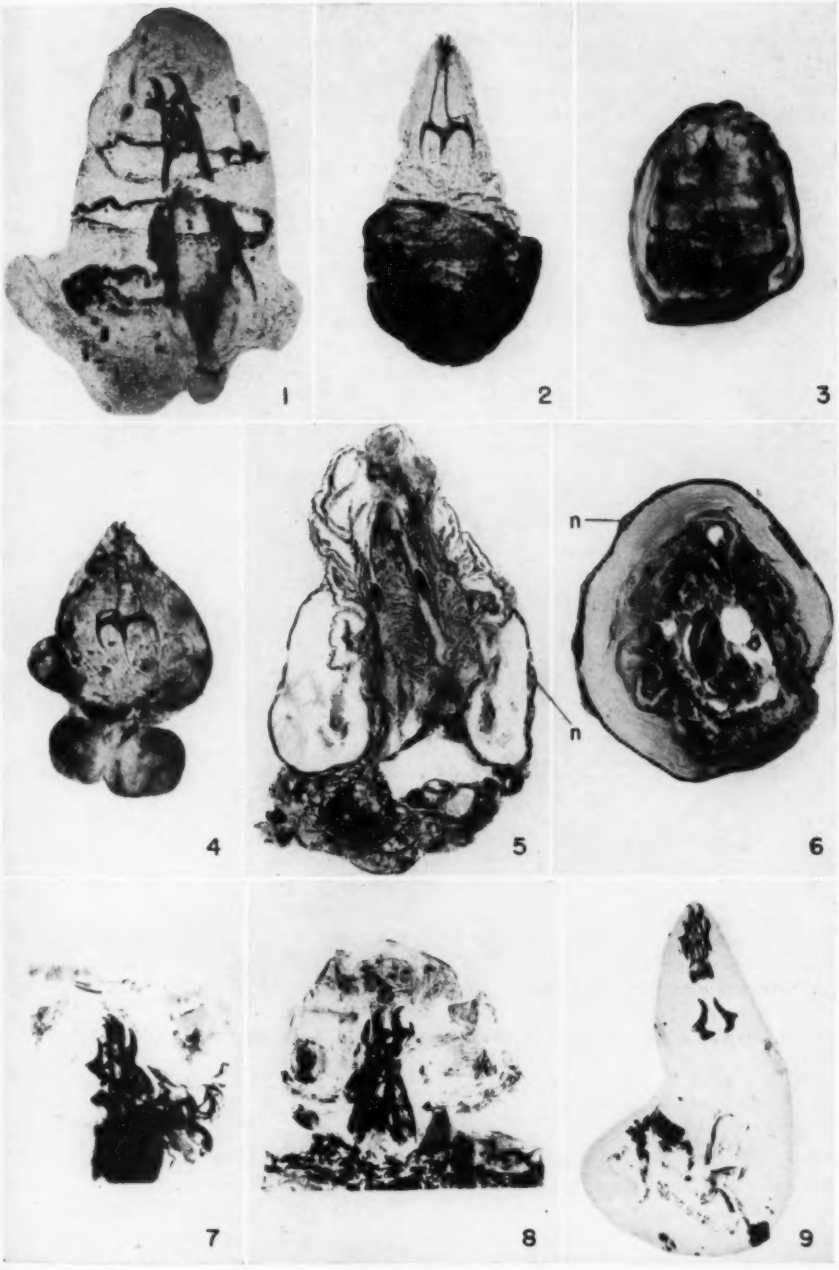

PLATE I

envelope also varies widely, from strictly larval color to darkish spotting or complete browning of the cuticle. Whether or not the browning of the cuticle is an indication of pupation remains questionable. A detailed histological study of the structure of the head parts has not been made. Thus too much emphasis cannot be placed on their apparently similar appearance.

It is interesting that the environment most favorable for molting is apparently that of melanogaster females. Three 1st instar head parts (Table II) which remained in melanogaster females for 7 days molted, while of six transplants which remained in virilis males or in virilis females for 7 to 8 days, only one molted (see Table II).

TABLE III

Transplantation of larval head segments into larval hosts

Age of transplanted larval head segments	Days transplant remains in host	Number of cases	Number of cases where graft has formed		
			1 pair of mandibles	2 pairs of mandibles	3 pairs of mandibles
1st instar (4 hrs. old)	5	6		4(1?)	1
1st instar (4 hrs. old)	6	1			1
2nd instar[a]	2	1	1		
2nd instar[a]	5	3	3		
2nd instar[a]	6	7		1(6?)	
2nd instar[a]	7	3		3	
2nd instar	5	1		1	
2nd instar	6	1		1	
2nd instar	7	6		6	
	Heteroplastic (virilis into pseudoobscura)				
1st instar (0–2 hrs. old)	5	1		1	
1st instar (0–2 hrs. old)	7	5			5
Total number of cases		35			24 positive

[a] Head skin removed, only mouth armature transplanted.

The transplantation of larval head segments into the abdomen of larval hosts

The object of this group of experiments was to test the molting behavior of larval head segments in a larval and pupal environment. For this the brain and ring gland were removed from head segments of 1st and 2nd instar larvae, which were then transplanted into the abdomen of 3rd instar host larvae. The transplanted pieces were dissected shortly before or after the emergence of the fly. Since the pupal life of virilis is about 5 to 5½ days, the head segments dissected 6 or 7

days after the operation were originally transplanted into larvae younger than those which were dissected 5 days after the operation. The head segments in these later dissected hosts therefore had remained longer in a larval environment. A few of the transplants were dissected from younger pupae. The results of these experiments are summarized in Table III, where it can be seen that molting occurred in the majority of the cases. Figure 8 shows such a case. All the head segments in this experimental combination reveal a dark brown cuticle which indeed resembles a pupal cuticle. Unfortunately, however, no special effort was made to determine

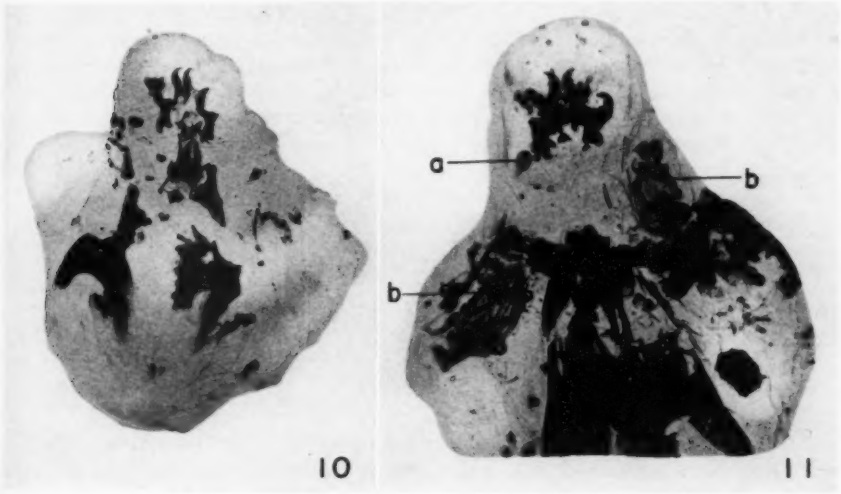

FIGURE 10. Head segments of 1st instar larva transplanted together with two larval ring glands into the abdomen of an adult melanogaster female; 7 days after the operation. The transplant has molted once. Note the two pairs of mandibular hooks.

FIGURE 11. Head segments of 1st instar larva transplanted together with three larval ring glands into an adult melanogaster female 8 days after the operation. The transplant has molted twice. Note the two pairs of mandibular hooks and the spiracles of the 2nd instar (a) and of the 3rd instar (b).

whether or not any epidermal overgrowth surrounded the transplant. Some of the 2nd instar head segments were transplanted after the skin around the mouth parts was removed, with the exception of a very small piece of skin left near the distal end. Such naked mouth parts are also able to molt (see Table III).

The molting factor is not species-specific. This is demonstrated by the transplantation of head segments of 1st instar virilis larvae without brain and ring gland into 3rd instar pseudoobscura larvae. From six cases available in this series, five had formed three pairs of mandibular hooks, revealing that they had molted twice (Fig. 9). One transplant, however, dissected somewhat earlier, had molted only once (see Table III).

The growth of young organ discs in adult hosts

Some of the organ discs such as eye, legs and antennae for example, may be left behind in the transplant after the removal of the nervous system, for these organ anlagen are too minute in size to be recognized in the dissection of the 1st instar larval transplants. Nothing has been said about their developmental fate while they remained in the adult fly. Larval organ anlagen are unable to develop in the abdomen of adult male hosts in the absence of larval ring glands (Bodenstein, 1943). Nevertheless we find in the experiments reported on page 114 that young eye discs left behind in the anterior part of 1st instar larvae develop quite extensively when transplanted into adult male hosts, although no larval ring glands were present. By their growth the discs are usually forced out through the cut surface of the small transplant so that they are finally placed almost or entirely outside of the implant itself. In Figure 4 are shown the two developed eye discs of a transplant which had remained for 14 days in the adult male host environment.

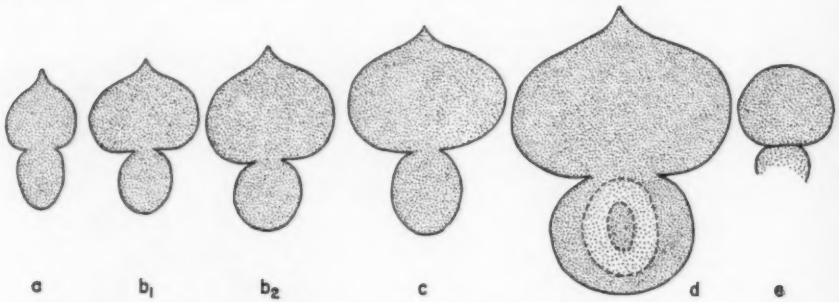

FIGURE 12. a. Eye-antenna disc of a 2nd instar larva shortly before the second molt. b_1 and b_2. Eye-antenna disc which had been transplanted into an adult male host at stage a. Note the increase in growth. c. Eye-antenna disc of very young 3rd instar larva. d. Eye-antenna disc of mature larva. e. Eye disc which had been transplanted at 1st instar stage into an adult male host. (For further explanation, see text.)

Since we know that eye discs of 3rd instar larvae are unable to develop in adult male hosts and find, as described above, that eye discs of 1st instar larvae are able to develop in the same environment, there must be one definite stage in development beyond which the discs are unable to develop. In order to find this critical stage one further experiment was designed and is semi-diagrammatically represented in Figure 12. The size relationships in this figure are based on camera lucida drawings of the same magnification. Figure 12 a shows an eye disc of a 2nd instar larva shortly before the second molt. One may observe that at this stage the distal portion of the disc, the eye disc proper and the posterior portion, the antenna disc, are separated only by a small constriction. Although the eye and antenna discs are bound up together during the whole of the larval life, they become more distinctly separated from one another as development proceeds. A few hours after the second molt the eye and antenna discs have become two definite structures, as illustrated in Figure 12 c. At this stage there is no differentiation recognizable in the antenna discs in total mounts. The eye disc of a mature larva

is shown in Figure 12 d. The considerable increase in size of the eye during the third larval instar is evident if one compares Figure 12 c with Figure 12 d. Moreover, histological differentiation has taken place during this time in both discs. In the mature eye discs the cells have arranged themselves into a definite pattern, which is however scarcely perceptible in total mounts, whereas the differentiation processes in the antenna are visible as oval concentric folds within the disc.

If eye discs of young 3rd instar larvae (Fig. 12 c) are transplanted into the abdomen of adult male flies it is noticed that these discs are unable to grow further. Yet eye discs of old 2nd instar larvae (Fig. 12 a) transplanted in the same manner reach the stage of growth shown in Figure 12 b_1 and b_2 but apparently do not develop further. In comparing the discs in Figures 12 b_2 and c, it may be noticed that both discs are almost the same size. From the foregoing consideration this should be expected, for since the discs of stage "c" are unable to grow in an adult male environment, the younger discs should cease growing in this environment when they reach stage "c." Thus the critical period for the cessation of growth of the eye discs lies between stages "a" and "c," i.e. at about the time the larvae undergo their second molt. Therefore the growth of eye discs transplanted at stages younger than "a" (see experiments above) should never surpass the growth stage "c." This expectation has been fulfilled, for one finds (Fig. 12 e) that the final growth stage of the largest eye disc shown in Figure 4 lies somewhere between the stages of growth represented in Figures 12 a and b.

Discussion

The results described in this paper emphasize the important part played by the ring gland in the larval molting of Drosophila. The molting of larval parts *in vivo* in the abdominal cavity of adult flies is possible only when larval ring glands are added. The speed with which the molts occur is, however, much slower than in normal development, where the two larval molts are accomplished during 4 days. In only one case has the transplant molted twice during the 7 days it remained in the adult host, while the majority of the cases molted only once, although some of them had remained as long as 15 days in the adult host. The slow developmental progress of larval structures in an adult environment was also observed in earlier experiments, and is apparently due to the different metabolic situation within the adult environment (Bodenstein, 1943). In this connection it is of interest to note that the transplant, which had molted twice, was a virilis head part transplanted together with two ring glands into a melanogaster female. It has been established (Bodenstein, 1943) that the melanogaster female environment is much better suited for the growth of organ discs than either melanogaster male or virilis female or male environments. It seems that the same applies to the molting of larval structures. This observation supports a suggestion (Bodenstein, 1943) that the ring gland does not act directly, but rather indirectly in affecting the metabolic level of the host, which then in turn is responsible for the various developmental achievements of the transplanted structures. This hypothesis also assumes that the larval or pupal level is much higher than the highest level induced by ring glands in the adult host (see Bodenstein, 1943, p. 56). If this is correct, it should follow that larval head parts of the first instar should always molt twice in a larval or pupal environment, and that the successive molts should proceed in more rapid succession

in a larval than in an adult environment. The fast rate of normal development, as compared with the very slow developmental rate of the transplanted head parts in adult hosts, is indicative in supporting the "level" conception. The experiments in which larval head segments were transplanted into the abdomen of 3rd instar larvae offer another case in point; for three out of eight cases had already molted twice in 5 to 6 days, while five out of five cases had also molted twice in 7 days. Moreover, in these cases the transplants were under the influence of but one ring gland, that of the host larva, while in the adult hosts two ring glands were present. A similar situation is also found when one compares the second instar larval head transplants in larval and adult host environments. The fact that several transplants in adult hosts had not molted at all, although they had been in their hosts for a considerable length of time, may also be explained by the very low effective level of this environment.

Molting is a process involving many different structures. In normal development there is apparently a marked synchrony between the structural systems involved, in that all of these tissues are ready to perform their molting task at one set time. The same situation, it would seem, prevails in the different organ anlagen systems, as far as their reactions towards pupation are concerned. Yet different organ anlagen vary in their competence to respond to the metamorphosis hormone. These differences, however, are not apparent in normal development, but become noticeable in a modified hormonal environment (see Bodenstein, 1943, p. 55). As far as the molting of head segments in adult flies is concerned, we find the spiracles apparently reacting more readily to the ring gland hormone than the mandibles. For although the spiracles are more difficult to detect, we have cases where it is certain that the mandibles have not molted, while the spiracles have (see Table II). Thus the different systems involved in the process of molting possess different degrees of reacting competence (see Bodenstein, 1943, pp. 53–55).

The number of molts in Drosophila seems to be fixed at two, for 2nd instar head parts transplanted into 3rd instar larvae molt only once, while 1st instar head parts may molt twice although they have remained no longer in the host than the 2nd instar head parts. This seems to be quite unusual in the light of our knowledge of hormone-induced molting in insects (see review Bodenstein, 1942). It might, however, be that the mouth parts used here as a criterion for molting react with greater difficulty than some other structures.

The competence of larval discs

It has been stated previously that in the development of organ discs, the processes of growth as well as of differentiation are controlled by the same hormone. Whether growth or differentiation takes place, depends upon a definite relationship between hormone level and organ competence (Bodenstein, 1943). The observation that young organ discs are able to grow in adult male hosts, but only to a certain stage of growth, still further clarifies the hormone control of Drosophila development. It shows that young discs are very responsive, at least as far as the growth response is concerned, since older discs fail to grow in the same environment. Furthermore these young eye discs grow only to a stage reached by normal eye discs at the time the larva enters the 3rd instar. This fact is decisive, since it shows that the male environment is effective in inducing growth only and does so

solely in very responsive young discs. The question now arises, have these young discs only the competence for a growth response, or are they also able to differentiate under the influence of a very effective hormone level? The answer is provided in an experiment (Bodenstein, 1939a) where young eye discs were transplanted into the abdomen of mature larvae, and thus subjected to a very highly effective level, with the result that they did not reach imaginal completion. Since older eye discs transplanted in the same manner complete their imaginal differentiation in synchrony with the host, it follows that the young discs respond with growth, until they have reached the size above which they can respond with differentiation. By this time, however, the host organs are already so far advanced in their differentiation that the young discs are unable to catch up. In the light of these considerations we might ask what takes place in the eye discs at the time they reach their maximal growth in the adult male fly? The young discs must have acquired the competence to react with differentiation if the appropriate stimulus is present. They are now bi-potent; they react to a low effective level with growth, but might be induced to differentiate, provided the effective hormone level is high enough. That this is the case can be demonstrated experimentally, for an eye disc of a 3rd instar larva will grow considerably before differentiation begins in an adult male environment if supported by two larval ring glands (Bodenstein, 1943). The same eye disc transplanted into a mature larva will, however, cease to grow and begin to differentiate immediately, with the result that finally a small imaginal eye is formed (Bodenstein, 1939a; 1941). This experiment shows clearly that the response of the same organ disc varies according to the hormonal situation. Thus we may conclude that as the eye discs of 3rd instar larvae become older their competence to respond with growth gradually decreases, while at the same time the competence of the differentiation response increases. At the end of larval life the discs are able to respond only with differentiation.

It is of interest to note that our earlier results (Bodenstein, 1943) and those discussed here are confirmed by recent experiments of Vogt (1943). This author, working with *Drosophila hydei*, approached the problem in a different way experimentally. She used as host the rear part of a 3rd instar larva, after the anterior larval part had been removed by means of a ligature. Into these larval rear parts she then transplanted the eye discs of 3rd instar larvae and simultaneously the eye discs plus the nervous system of very young 2nd instar larvae. She found that the older eye discs remained larval (whether growth occurred is not stated) while the young eye discs developed to a stage equivalent to that of an old 2nd instar larva. When, however, she transplanted six ring glands of the young 2nd instar donors simultaneously, the result was different. The young eye discs had become larger, while the old eye discs had begun differentiation. From this Vogt concludes that growth and differentiation are apparently controlled by the same hormone.

Here attention should be drawn to the fact that this critical stage which occurs at about the time of the second molt corresponds closely to critical periods observed in two other sets of experiments. 1. Beadle *et al.* (1938) and Bodenstein (1939b; 1941) observed that Drosophila larvae (melanogaster and hydei) when starved completely before they are 50 and 100 to 120 hours old respectively, i.e. about the time of the second molt, are unable to pupate. 2. Bodenstein (1939a;

1941) transplanted melanogaster eye discs from donor larvae younger than 50 hours into mature larvae and found that these discs were unable to differentiate completely, while older eye discs transplanted in the same manner differentiate to imaginal completion. From all this it becomes evident that at this time a change occurs in the organ discs of the larvae, the nature of which we have discussed above. Whether all the imaginal discs reach this critical stage at the same time is questionable, since one must remember that the different discs, as well as different regions of the same discs (Bodenstein, 1943), respond differently to the same effective level. Whether all discs have such a critical stage is at present unknown, but seems highly probable.

Summary

1. Larval head parts of 1st and 2nd instar larvae without nervous system and ring glands were transplanted into the abdomen of adult male flies, together with two larval ring glands from mature larvae. The transplanted heads molt once or twice, as indicated by the presence of double or triple mouth parts.

2. Head parts transplanted in the same manner, but without larval ring glands fail to molt. This shows that the larval ring gland hormone induces larval molting.

3. Head parts without their nervous system and ring glands were also transplanted into the abdomen of 3rd instar larvae. The transplantations were performed either homo- or heteroplastically. In either case molting was induced. The molting hormone of the ring gland is thus not species-specific.

4. Very young organ anlagen were transplanted together with anterior larval head segments after the removal of the nervous system into adult male hosts. It was found that these organs were able to develop in their new environment in the absence of ring glands. Older organ discs transplanted in the same manner develop however only in the presence of ring glands. These experiments are discussed in relation to hormone level and tissue competence.

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THE DEVELOPMENT OF FUCUS EGGS IN CONCENTRATION GRADIENTS: A NEW METHOD FOR ESTABLISHING STEEP GRADIENTS ACROSS LIVING CELLS[1]

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Introduction

Fucus eggs have been reared in gradients of hydrogen ion concentration (Whitaker, 1938; 1940) and in temperature gradients (Lowrance, 1937), by methods already described. The developmental polarity of the embryo was determined in these cases, and further analysis by means of gradients of known substances and conditions is desirable. Pease (1941) has reared Echinoderm eggs in concentration gradients of enzyme poisons and other substances by placing them on a membrane of scintered glass that separates two solutions. He has been able to associate the determination of the dorso-ventral axis with parts of the cyanide-sensitive respiratory mechanism.

The present paper describes a simple new method for establishing very steep gradients across living cells. It is applicable to a wide variety of types of living cells, and the steep gradients may be either chemical or physical, including temperature and gas pressures as well as various water solutions. The method also permits a relatively exact estimate of the actual concentration or condition at all parts of the cell surface. In the experiments now to be reported, gradients of dinitrophenol were applied to Fucus eggs in August and September, 1941.

Method

The method consists essentially in dispersing living cells in a liquid medium that will gelate when the temperature decreases, and in dipping a ring-like collar of glass into the liquid so that a very thin film is formed in the collar, similar in general to a soap bubble film or to the film formed in a bacteriological loop. The film, which contains living cells, gelates on cooling. The collar is then fitted into a hole in a glass or lucite partition that separates two flowing solutions of different composition, and the thin film thus becomes a part of the partition. The concentration gradient in the film may be very steep because the film is very thin. Solutions at different temperatures, or gas mixtures, may be substituted for the solutions of different composition.

The procedure as actually carried out will now be described in more detail. A drop of sea water containing Fucus eggs [2] was mixed with a drop of 2 to 3 per cent agar-sea water in a small watch glass or other small glass vessel. At the time of the mixing the agar was fluid, but it was close to congealing temperature, having

[1] This work was supported in part by funds granted by the Rockefeller Foundation.

[2] Five to six hours fertilized, in these experiments.

cooled nearly to 37° C. The watch glass was warmed slightly before the eggs and agar were added so that the mixture would not congeal instantly.

Collar-like cylindrical cross sections of an ordinary 2 cm. glass tube had been cut earlier with a glass saw. Each of these glass collars or cylinders was about one-eighth inch long. The actual mixing of the eggs and agar, as described in the preceding paragraph, was done with a glass collar held in the fingers. The mixing must be done very rapidly, as the high temperature is damaging if maintained. The collar was pulled away quickly after the mixing, and this caused a very thin film of agar to form across and within the collar. It was not difficult to remove the collar within 1 to 2 seconds after mixing the eggs with the warm agar, and the thin film cooled almost instantly.

Many films were removed from the collars and thin sections of the films, cut with razor blades, were examined under the microscope. The main central area of a film was found to be quite uniform in thickness. Near the edge the film became suddenly thinner and then flared out into a thick attachment meniscus. The eggs were found to be fairly evenly distributed throughout the agar film, in which they are firmly embedded so that they do not roll or move. The thickness of the film could be varied, with a little practice, by varying the concentration of agar, the temperature of the mixture, and the speed of withdrawal of the collar. It was also found possible by successive dippings to build up a multiple laminated film. The thickness of measured single dipped films varied from 30 to 150 μ, and the range could readily have been extended in either direction. The Fucus eggs average about 80 μ in diameter, and in very thin films they bulged out of the film on both sides. They remained firmly embedded however, and it is therefore presumed that a very thin film of agar covered the bulging surfaces of the eggs. In the experiments to be reported below, it was arranged for the films to be as thick as or slightly thicker than the diameter of the eggs. The films ranged from 80 to 130 μ thick.

Gelatin and gelatin-agar mixtures were also used for films. Gelatin has the advantage of a lower congealing temperature but the gelatin available to us (bacteriological) tended to be somewhat toxic to the Fucus eggs, and the experiments were therefore carried out in agar. Other gels might well turn out to be quite satisfactory.

Once the eggs were embedded in the film in the collar, to subject them to steep gradients it was necessary only to place the film as a sort of window in a partition separating two different solutions. This was done by drilling a 2 cm. hole in a glass plate and grinding one of the collars to fit snugly into the hole. The collar was mounted into the plate before dipping the film and it was to some extent cemented in place with agar-sea water jelly which filled any imperfections in the ground fit. The upper half of a Coplin jar was cut off and discarded. The glass plate bearing the collar (containing the film) was inserted into the lower half of the Coplin jar thereby dividing it into two chambers. The plate fitted in between the fins or corrugations originally designed to hold slides in the Coplin jar. The plate had been cut to fit the slightly tapered jar, and one or more wide rubber bands were snapped around the edge of the glass plate before it was inserted in the Coplin jar. These rubber bands acted as an elastic gasket that sealed the fit when the plate was pushed firmly down into the jar.

It was necessary to maintain equal pressure on the two sides of the film to prevent its rupture. The lower half of the Coplin jar was filled with sea water before the plate was carefully pushed into place. Sea water was then caused to flow into the chamber on one side of the plate, while the experimental solution (e.g. dinitrophenol in sea water) flowed into the chamber on the other side of the plate. The rates of flow were adjusted by means of stopcocks to be about the same on both sides, to minimize pressure difference, and each chamber was drained by means of a levelling siphon leading in both cases into the same small dumping reservoir. The dumping reservoir was a small vial held to the side of the Coplin jar with a rubber band so that its height could readily be adjusted. It drained by overflowing.

Several duplicate apparatuses were set up at the same time, and the two solutions were siphoned into the chambers from large reservoirs. The rate of flow into the chambers was judged to be more than ample to maintain the concentrations in the chambers and at the surfaces of the agar film in spite of diffusion through the film. Concentration at any distance into the film can be estimated on the basis of calculated diffusion.

Fucus eggs embedded in an agar film develop normally when the film is maintained in a moist atmosphere instead of in sea water. The agar gel serves as an adequate medium provided it is prevented from drying out. It is thus possible to subject living cells to concentration gradients of gasses in the agar film by flowing moist gas mixtures through chambers on either side of the film.

In certain later experiments lucite plates were used instead of glass. The lucite was non-toxic and was easier to cut and drill than glass. Rectangular glass vessels were sawed in half across the middle of the long axis, and a lucite plate with flat rubber gaskets was placed between the two halves of the vessel, which were pressed firmly against the plate so that there was no leakage. Solutions flowed into the two halves of the vessel, now serving as the two chambers on either side of the plate.

All experiments were set up in dim red light, after which the Fucus eggs were reared in the dark until the end of the experiments since the point on the surface of the egg at which the rhizoid forms is affected by the shorter wave-lengths of the visible spectrum. The eggs were kept at 15° C., except during the embedding process.

Results

After the embedded Fucus eggs had been fertilized 24 to 30 hours, the glass plates were removed from the Coplin jars and placed flat in small dishes containing sea water. The film was inspected microscopically to make counts of rhizoids pointing up or down, i.e. toward or away from the surface of the film facing the lens of the microscope. After a count had been made the plate and film were turned over and a count was made from the other side as a check. Counts made from the two sides were averaged. They usually agreed quite well. In some cases thin strips of the agar film bearing eggs were cut with a razor, and these strips were then examined on edge under the microscope to observe the direction of rhizoid formation and growth.

In the present experiments the agar films were 80–130 μ thick. The pH of the media ranged in different experiments within the limits: 8.2 ± 0.05. A stock solution of dinitrophenol (10^{-3} M) in sea water was adjusted to the pH of the prevail-

ing sample of sea water before being added into the final sea water-dinitrophenol solution. The pH of sea water and dinitrophenol-sea water did not differ measurably in an experiment.

The results are shown in Table I. The controls, with sea water on both sides of the film, serve to indicate the accuracy of counting since the control counts should approximate 50 per cent toward one side. It is clear that a high proportion of the

TABLE I

The percentages of rhizoids formed on the sides of eggs subjected to the highest concentration of dinitrophenol are shown in the right hand column. The controls indicate the accuracy of the method of counting, since approximately 50 per cent of the rhizoids in the controls would be expected to form toward one side of the membrane

Experiment number	Concentration dinitrophenol M.	Number of eggs	Rhizoids toward dinitrophenol %
1	5×10^{-5}	119	95
2	4×10^{-5}	124	99
3	4×10^{-5}	58	98
4	3×10^{-5}	123	99
5	3×10^{-5}	79	87
6	3×10^{-5}	108	73
7	3×10^{-5}	175	95
8	3×10^{-5}	92	74
9	3×10^{-5}	132	95
10	3×10^{-5}	101	79
			Rhizoids toward one side, %
Control	0	104	53
Control	0	55	51
Control	0	70	58

rhizoids formed on the sides of the eggs subjected in the gradients to the higher concentrations of dinitrophenol.

Experiments have not yet been carried out to show precisely the effects of dinitrophenol on the respiration of Fucus eggs, but on general grounds it might be expected to affect respiratory rate.

SUMMARY AND CONCLUSIONS

1. A new method is described for establishing across living cells very steep concentration gradients of chemical substances in solution, or for establishing temperature gradients. The cells are embedded in a thin jelly membrane that separates two different flowing solutions.

2. When fertilized Fucus eggs, embedded in a thin agar membrane, are reared with sea water on one side and $3–5 \times 10^{-5}$ M dinitrophenol in sea water on the other side of the membrane, a high proportion form rhizoids on the sides toward the higher concentration of dinitrophenol.

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